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ADRENAL WEIGHT-MAINTAINING FACTORS OTHER THAN ACTH IN A BEEF PITUITARY EXTRACT

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ABSTRACT

It has been reported that an alkaline extract of beef pituitary glands contained an adrenotropic factor which was potent in influencing adrenal weight but relatively poor in causing adrenal ascorbic acid depletion. We have prepared and tested such an extract and believe that our preparation contained at least two types of activity, each capable of maintaining adrenal weights in hypophysectomized rats. These two types of activity appeared to be identifiable with the activities of two recognized pituitary hormones: luteinizing hormone (LH) and growth hormone (GH). No dissociation of ACTH into an adrenal weight maintaining factor and an adrenal ascorbic acid depleting factor need be postulated to explain our results.

WHEN various preparations of ACTH are compared, equivalent amounts as measured by the ascorbic acid depletion method do not always produce equivalent results in adrenal weight maintenance tests (1, 2, 3). Such observations have led Young (4) to suggest that ACTH may be composed of two factors, one causing ascorbic acid depletion (AA) and one influencing adrenal weight (AW). We have been interested in the postulated AW factor because of our interest in the human fetal adrenal, which is strikingly enlarged by adult standards during *intrauterine* life. The factor responsible for its prenatal growth is unknown, and we believed that a greater knowledge of the AW factor might suggest the nature of the tropic influence responsible for fetal adrenal enlargement. Dasgupta and Young (5) reported that an alkaline extract of beef pituitaries was rich in AW and relatively poor in AA. We have therefore attempted to determine the nature of the AW factor in such an extract. We believe that we have evidence

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of at least two types of activity in our preparation, each of which was capable of maintaining adrenal weight in hypophysectomized rats. These two types of activity appeared to be identifiable with the activities of two recognized pituitary hormones: luteinizing hormone (LH) and growth hormone (GH). We believe it is therefore unnecessary to postulate any separation of ACTH into AW and AA factors in order to explain the action of our alkaline extract of beef pituitaries on the adrenal.

METHODS

Preparation of Pituitary Extract (PE). Approximately 900 grams of beef pituitaries were frozen promptly on removal from the animals. The material was subsequently kept cold until the final frozen-dried product was obtained. The anterior lobes were separated manually from the posterior lobes after partial thawing, yielding 500 gm. of anterior lobe tissue. This was ground in sand and 1 liter of normal saline. The pH was adjusted to 8.5. The material was centrifuged, and the supernatant filtered through a Buchner funnel. The filtrate was recentrifuged at 9000×G and the supernatant dried in a freeze-dryer, yielding 15.8 gm. of powder. The material was stored in a desiccator until used. The abbreviation PE applies only to this preparation.

Ascorbic acid depletion assays were run by the method of Sayers and coworkers (6); hypophysectomized rats were obtained from the Hormone Assay Laboratories, Chicago. Gonadotropin assays were made by the ovarian hyperemia method of Albert and Berkson (7). Adrenal weight maintenance tests were run in 40-day old male hypophysectomized and hypophysectomized, castrate rats of the Charles River strain, Caesarian derived, obtained from the Charles River Breeding Laboratories. Each rat received a subcutaneous injection of 0.3 cc. three times daily (approximately 9:30 A.M., noon, and 4 P.M.) for eight days, beginning two days after operation. The animals were then sacrificed and the adrenals weighed and then fixed in formalin for histological section.

In all animal experiments, animals were assigned to treatment groups and (where appropriate) to individual cages by random numbers, and results were obtained without knowledge of the treatment involved. Each treatment group contained 5 to 7 animals.

Chorionic gonadotropin (CG) was a commercial preparation obtained from human pregnancy urine and kindly supplied by Dr. A. Stanley Cook of Ayerst Laboratories. It contained 1225 I.U./mg. CG activity and less than 0.3 MU./mg. ACTH activity as measured by ascorbic acid depletion. ACTH was kindly supplied by the Upjohn Co. GH was a highly purified bovine preparation supplied as a gift by the Endocrinology Study Section of the National Institutes of Health. Data supplied with the GH (NIH-BGH-1), indicated that it contained less than 0.06 MU. (U.S.P.)/mg. ACTH, and no detectable LH activity when tested in immature male rats (total dose, 15 mg. over 4 days).

RESULTS

Assay of PE by the ovarian hyperemia method revealed that 5 mg. contained activity equivalent to that of 7.5 I.U. CG (95% confidence limits: 4.6–12.5 I.U. CG ≏). ACTH activity of PE by the ascorbic acid depletion assay was equivalent to 0.8 MU./mg; after the preparation had stood for 30 minutes in an aqueous solution acidified to pH 3.0 with HCl, its ACTH activity was 1.8 MU./mg. This difference was not significant at the 5% level.

Results of adrenal weight maintenance studies, together with associated body weight changes, are shown in Table 1. Each of the two experiments was performed on a single lot of randomized animals treated simultaneously. In experiment A (Table 1) hypophysectomized rats were used. PE was given in a dose of 5 mg., an amount equivalent to 7.5 I.U. CG and 4 MU. ACTH. This dose produced a significant increase in adrenal weight over control values. A similar increase was obtained with 9 I.U. CG, but 4.5 MU. ACTH failed to produce a significant change. PE produced body weight increases of 4.7 gm./day. None of the other treatments were associated with weight changes of this order of magnitude.

In Experiment B (Table 1) using hypophysectomized, castrate rats, GH

TABLE 1. THE EFFECT OF PITUITARY EXTRACT (PE), CHORIONIC GONADOTROPIN (CG), ACTH, AND GROWTH HORMONE (GH) ON ADRENAL AND BODY WEIGHTS OF HYPOPHYSECTOMIZED AND HYPOPHYSECTOMIZED-CASTRATE MALE RATS

Experiment	Material	Dose/rat/day	Adrenal mean	Weight (mg.) std. error of mean	Change in body weight (gm./day)
(A)					
Non-castrate	Normal saline	0.9 cc.	11.4[a]	0.9	0.0
	PE	5.0 mg.	20.8[b,c]	1.0	+4.7
	CG	9 I.U.	17.8[b]	0.7	+0.7
	ACTH	4.5 MU.	13.6[d]	0.8	−0.5
(B)					
Castrate	PO_4 buffer (pH 6.85)	0.9 cc.	11.4[e]	0.5	−1.1
	PE (pH 6.85)	5 mg.	18.3[f]	0.8	+4.7
	PE (pH 3.0)	5 mg.	16.7[f]	0.6	+1.9
	GH (pH 6.85)	50 μg.	16.3[f]	1.0	+1.1
	GH (pH 3.0)	50 μg.	16.4[f]	1.0	+0.5

a–b; c–d; e–f $P < 0.01$.

and PE both produced significant increases in adrenal weight over control values. Both also produced increments in body weight, although the increment with PE (4.7 gm./day) was considerably greater than that produced by 50 μg. GH (1.1 gm./day). This dose of GH produced an unexpectedly small effect on body weight in these studies. We believe this was caused by the insolubility of the preparation. Despite various attempts, no clear solution could be attained, and we believe the physiologic activity may have suffered. PE and GH in aqueous medium acidified to pH 3.0 at least 30 minutes before administration had approximately the same effect on adrenal weight as when given at pH 6.85, but produced less of an increase in body weight (1.9 and 0.5 gm./day respectively).

Histological sections of the adrenals stained with Sudan revealed a sudanophobic zone beneath the glomerular zone in glands from animals treated with PE (Fig. 1), CG, ACTH in the dose used, and GH (Fig. 2) as well as in control animals. Sections of testis showed that the interstitial cells

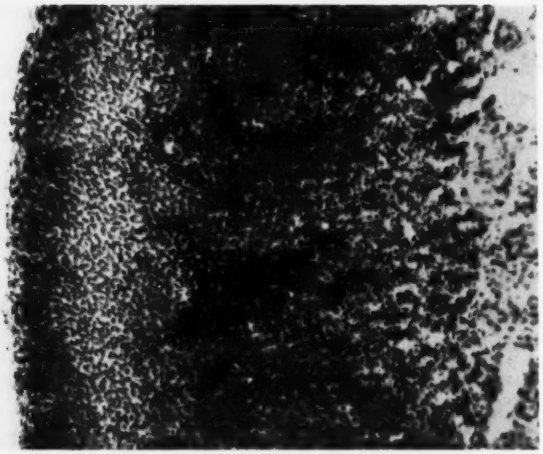

FIG. 1. Treatment with an alkaline extract of beef pituitaries failed to eliminate the subglomerular sudanophobic zone in the adrenals of hypophysectomized male rats. (Sudan stain, ×128)

were prominent in animals treated with PE and CG, and were atrophic in control animals.

DISCUSSION

We have sought an explanation for the adrenal weight maintaining potency of a crude beef pituitary extract (PE) which appeared to contain little ACTH as measured in the adrenal ascorbic acid depletion assay. Hor-

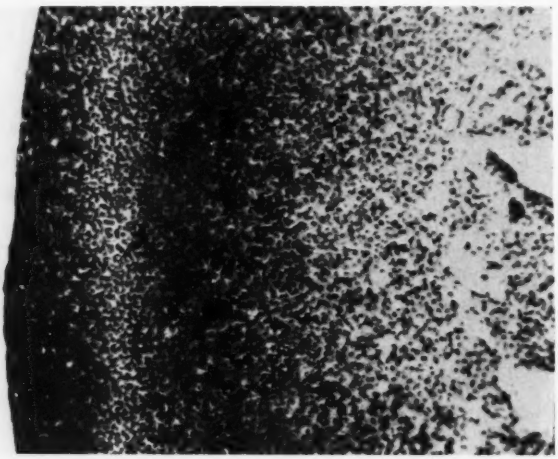

FIG. 2. Treatment with growth hormone failed to eliminate the subglomerular sudanophobic zone in hypophysectomized male rats. (Sudan stain, ×128)

mones that were possibly present in the crude extract and that have been reported previously to influence adrenal weight were (1) ACTH, (2) gonadotropins and (3) GH. We believe we have shown that ACTH contained in PE was not responsible for the observed effects, but that PE contained sufficient gonadotropin and GH to influence adrenal weights to the extent demonstrated.

ACTH: The ACTH content of PE measured in the ascorbic acid depletion assay was insufficient to produce the observed changes in adrenal weight. In the dose given, PE contained 4 mU. ACTH activity; previous work (8) has shown that the minimum dose required to increase adrenal weight significantly in our experimental procedure was between 25 and 75 mU. However, it has been postulated that ACTH may exist in a form potent in influencing adrenal weight but relatively poor in ascorbic acid depleting potency. Young believed that such an AW factor existed in the alkaline beef pituitary extract. In his studies (9), hypophysectomized rats treated with this material had adrenals in which a sudanophobic zone was lacking. This zone appears in the adrenals of hypophysectomized rats and is believed to disappear only under the influence of ACTH; it is a more sensitive indicator of the presence of ACTH than is the adrenal weight (10). In our studies of animals treated with PE, a sudanophobic zone was present, suggesting that ACTH could not account for the adrenal changes in our animals. The cause for this difference between Young's pituitary extract and our own was not apparent; as far as we know, it was the only instance in which different results were obtained where parallel observations have been made with the two preparations.

Dasgupta and Young (5) have recently reported that the ACTH content of their alkaline pituitary extract as measured by ascorbic acid depletion could be increased by treatment with acid. We have some evidence of a similar increase on acid treatment of PE, although the difference was not significant at the 5% level. However, the ACTH content of the acid-treated material was still below the amount required to influence adrenal weight, and, on histological examination, a sudanophobic zone was again present. We believed, therefore, that neither ACTH nor a possible precursor could explain the adrenal changes seen in these studies.

Gonadotropin: The mechanism by which CG and other gonadotropins with LH activity influenced adrenal weight has been previously clarified (8). The gonadotropins did not act directly on the adrenal. Instead, they stimulated the interstitial cells of the testis to secrete a product which acted on the adrenals to maintain their weight in freshly hypophysectomized male rats. In castrate, hypophysectomized rats, gonadotropins were ineffective, but in these animals testosterone propionate maintained adrenal weight. The maximum effect of gonadotropins and testosterone appeared to be a partial prevention of involution of the adrenal (Fig. 3). We have no evidence that these preparations increased adrenal weight in

freshly hypophysectomized rats, and neither CG nor testosterone was capable of significantly increasing adrenal weights of hypophysectomized rats in which the adrenal had been permitted to involute before treatment was begun.

PE contained sufficient material with gonadotropic activity to produce the observed changes in adrenal weight in hypophysectomized male rats. However, PE was equally effective in hypophysectomized, castrate rats, a test animal in which gonadotropins were ineffective. This finding suggested that a second adrenotropin was present. With PE containing two

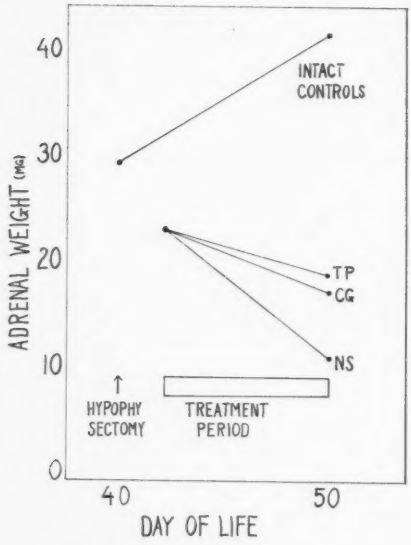

FIG. 3. The partial prevention of adrenal involution in hypophysectomized male rats treated with chorionic gonadotropin (CG) and testosterone propionate (TP) as compared with control animals treated with normal saline (NS) and with intact control animals.

possible factors acting simultaneously, we were unable to determine whether the gonadotropin was the actual cause of adrenal weight maintenance in the non-castrate animals, though we believe that the gonadotropin was potentially capable of producing the observed adrenal weight response and must be regarded as an adrenal weight-maintaining factor in PE.

Growth Hormone: Our attention was directed to GH as a possible second adrenal weight factor in PE because of the striking weight gains in hypophysectomized animals treated with this material. Several investigators have found that various preparations of GH influence adrenal weight (9, 11, 12) and we have confirmed this in castrate, hypophysectomized male rats, a test animal in which gonadotropins were ineffective. Li (11) believed that the effect of GH on adrenal weight was usually less than that which would have been expected from a proportionate participation of the adrenal in the general body weight increase caused by GH. He found that after prolonged

treatment the absolute weights of the adrenal were fairly uniform despite differences in total body weight produced by variations in the length of treatment. In our studies on freshly hypophysectomized rats, the effect of GH appeared to be largely one of maintenance of adrenal weight at approximately the level found at the beginning of treatment (Fig. 4); we have found in other experiments that GH had a limited capacity to increase adrenal weights in hypophysectomized rats with already atrophic adrenals, but we have not been able to produce adrenal weights in excess of those found at the time of hypophysectomy. We have examined our results with

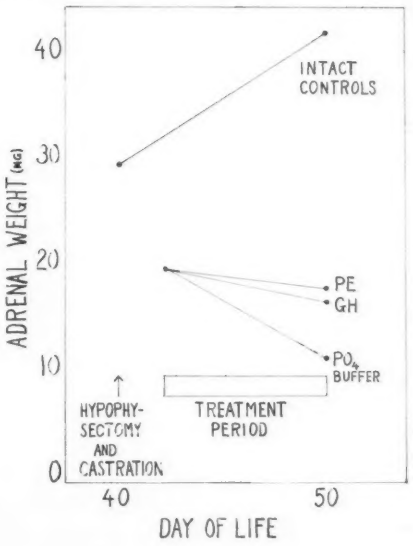

FIG. 4. The partial prevention of adrenal involution in hypophysectomized male rats treated with growth hormone (GH) and an alkaline extract of beef pituitary glands (PE) as compared with control animals treated with phosphate buffer and with intact control animals.

adrenal weights expressed as mg./100 gm. body weight and have not been led to a different interpretation of the effect of GH on the adrenal.

Young has suggested that growth hormone preparations carry a potentially separable component influencing adrenal weight (AW). Thus far, attempts to separate AW from GH have failed (13) and we believe that at present the adrenotropic effect must be regarded as an effect of GH itself.

Conclusions regarding the effect of GH on the adrenal appeared to be applicable to PE, since PE gave evidence of containing GH by producing large weight gains in hypophysectomized rats. The effects of PE on the adrenal were similar to those of GH; both failed to eliminate the adrenal sudanophobic zone and both produced adrenal weight changes of similar magnitude in hypophysectomized, castrate rats.

The evidence presented led us to believe that there were two adrenal weight-maintaining factors in our alkaline extract of beef pituitaries. These were a gonadotropin with LH activity and GH. In our studies the effect of

gonadotropin and of GH was one of maintenance of adrenal weight, and, when the two were given together in PE, no further increase was obtainable. There was no evidence that ACTH or a possible precursor was present in sufficient amount to influence either adrenal weight or histology, and it appeared unnecessary to postulate that ACTH was separable into an AA and AW factor. Similarly, there was little evidence to suggest that GH was dissociable into factors separately influencing body weight and adrenal weight. It appeared unlikely that either GH or gonadotropins acting in the manner found in these studies could be important in producing the striking enlargement of the adrenal gland which occurs normally in the human fetus.

Acknowledgment

We wish to acknowledge the help of Mrs. Lee Herrera of the Department of Medical Statistics, New York University, in interpreting these studies.

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THE CORPUS LUTEUM-PITUITARY RELATIONSHIP: THE ASSOCIATION BETWEEN THE CAUSE OF LUTEOTROPHIN SECRETION AND THE CAUSE OF FOLLICULAR QUIESCENCE DURING LACTATION; THE BASIS FOR A TENTATIVE THEORY OF THE CORPUS LUTEUM-PITUITARY RELATIONSHIP IN THE RAT[1]

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ABSTRACT

The regulation of pituitary luteotrophin (LTH) secretion was studied by investigating a) the association between LTH secretion and follicular quiescence in lactating rats, and b) the maintenance of lactation in rats bearing denervated pituitaries. Follicular quiescence was not due to an LTH effect on the ovary, since a) exogenous LTH did not alter the mouse uterine weight response to exogenous folliculotrophins (FSH and LH; abbreviated as FTH), nor the ovarian autograft response of castrated rats to endogenous FTH, and b) pseudopregnant and lactating rats' ovaries responded to exogenous FTH as well as those of cycling rats. That follicular quiescence was therefore probably due to FTH suppression was confirmed by the following. Ovarian autografts in castrated lactators with large litters grew much less than did those of castrated post-parturient rats deprived of their litters. Castration did not increase the pituitary FTH potency of lactators nursing large litters. That FTH suppression was proportional to litter size was shown by the following. The interval to first estrus in intact lactators and in castrated lactators bearing ovarian autografts increased with the size of the litter; ovarian autotransplant growth increased with decrease in litter size. Castrates deprived of their litters and castrates nursing 2-pup litters showed the same increase in pituitary FTH potency. That FTH suppression did not cause LTH secretion was shown by the following. Intact and castrated lactators with small litters showed very little or no suppression of FTH, but secreted enough LTH to maintain milk secretion for normal pup growth. That LTH secretion was not continuous was shown by the facts that about 50% of rats deprived of their litters shortly after delivery became pseudopregnant, while 100% of rats suckling 1-pup litters became pseudopregnant (other evidence supporting the discontinuity of LTH secretion was also cited). Lactation was maintained in oxytocin-treated hypophysectomized rats bearing pituitaries autotransplanted beneath the kidney capsule; oxytocin was shown in another study not to stimulate LTH secretion. This experiment thus demonstrated that LTH secretion did not depend on stimulation from the CNS. These results appeared to support a hypothesis that a CNS influence inhibits

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LTH secretion, that another CNS influence stimulates FTH secretion, and that suckling suppresses both CNS influences. An attempt was made to apply the principle of this explanation to an analysis of pseudopregnancy and the corpus luteum-pituitary relationship in the rat.

THE ultimate aim of these studies is the understanding of the corpus luteum-pituitary relationship and its connection with the regulation of the ovulation cycle. The determination of the factors that control luteotrophin secretion, and those that limit the viability of the corpus luteum, was considered essential to this. In the course of this and other studies on the regulation of luteotrophin secretion, clues to some of the factors that affect corpus luteum viability appeared, some of which will be described here briefly. Their detailed presentation, however, will be left for a later paper.

The definition of several terms at this point will be of help in this and other papers. Luteotrophin (LTH) is used throughout as synonymous with prolactin or lactogenic hormone; detailed reasons for this choice may be found in White (1). FSH and LH are designated by the generic term "folliculotrophins," for which the abbreviation FTH is used. The purpose of this is to facilitate discussion of the differences between the folliculotrophins and LTH in their relations to both the central nervous system (CNS) and their target organs, without having to specify which of the two hormones is involved, and in what proportion with the other. The generic term is a desirable and necessary tool for discussion, since in many instances specificity in relation to these problems is impossible. The avoidance of the term "hypothalamus," in reference to the CNS is purely intentional. The postulated CNS influences over FTH and LTH secretion may be in the hypothalamus, but until it can be shown that no other part of the CNS is involved in this control, it is best to avoid specificity here as well.

The experiments described in this paper were undertaken primarily to determine how the secretion of LTH is regulated. Most of them attacked the possible causes of the follicular quiescence that accompanies LTH secretion in the rat during lactation. The reason for this was as follows. Follicular quiescence during lactation could be due either to the inhibiting effect of LTH (or the conditions under which it is secreted) on the ovary's response to FTH stimulation, or to the suppression of FTH secretion. If the latter explanation is correct, three possible reasons may be postulated for its association with LTH secretion: 1) the stimuli that lead to LTH secretion also cause suppression of FTH secretion; 2) the suppression of FTH secretion itself directly causes the secretion of LTH; and 3) LTH is always being secreted, but the factors that appear to stimulate if actually cause only the suppression of FTH secretion. The cause of FTH suppression (provided this is the actual basis for follicular quiescence) could thus be a clue to understanding the regulation of LTH secretion, since the first two of

these reasons relate the cause of this suppression to a specific cause of LTH secretion.

This aspect of the problem was therefore attacked along the following two main lines: a) tests to determine whether LTH, or the conditions under which it is secreted, would inhibit the ovary's response to FTH stimulation; and b) a study to determine whether quantitation of the suckling stimulus, through variations in litter size, would elicit differential effects on FTH and LTH secretion in lactating rats.

Another aspect of the problem involved the ability of the pituitary autotransplanted to the kidney to secrete LTH (2, 3); this suggested that suckling does not directly stimulate LTH secretion, but rather permits it to occur, possibly by suppressing the activity of a CNS inhibitor of its secretion. Everett has suggested (2, 3) that such a CNS influence might account for the behavior of the autotransplanted pituitary. The ability of hypophysectomized rats bearing autotransplanted pituitaries to lactate was therefore tested in another series of experiments.

For the sake of clarity of presentation, the discussion of each experiment or set of experiments will be incorporated with the description of the results. The significance of the results of these studies, together with others available in the literature, for an interpretation of the regulation of LTH secretion in general, and the corpus luteum-pituitary relationship in particular, is considered in the General Discussion.

METHODS AND MATERIALS

General experimental plan. The effect of LTH on the ovary's response to FTH was tested by observing 1) the uterine weight increase of 21 day old female mice in response to combinations of LTH and FTH; 2) the interval from castration to the first postoperative vaginal estrus in LTH-treated rats bearing autotransplanted ovarian tissue; and 3) ovarian growth in response to injected FTH in intact lactating, pseudopregnant, and cycling rats.

The effect of litter size on the following end points of FTH secretion was studied: 1) the interval from delivery to the first vaginal estrus in intact lactating rats; 2, a) the interval from castration and ovarian autotransplantation, to the first postoperative vaginal estrus, and b) the growth of the ovarian autotransplants in these castrated lactating rats; and 3) the effect of castration on the FTH potency of the pituitaries of lactating rats.

The ability of the denervated pituitary to maintain lactation was studied in lactators subjected to hypophysectomy and pituitary autotransplantation. The details of operation and further treatment, and the use of pup growth as an index of lactation, are given below.

General. All rats were 200 to 300 gm. Sprague-Dawley females (Holtzman Rat Company, Madison Wisc.). Pregnant females were placed in isolation cages about 3 days before term and given newspaper scraps for nest building. Once or twice daily observations for the time of delivery were made; day 1 postpartum was taken as the first day *after* delivery. The lactating females received no special care, except for isolation, and were not handled until day 1 or 2 postpartum. At this time they were weighed, and

their litters were counted and weighed. From this time until the end of each experiment, with very few exceptions, daily vaginal smears were made on each mother, at which times her litter was also counted, weighed, and observed for evidence of milk ingestion.

Cycling and pseudopregnant rats were selected and maintained for specific experiments under the conditions, criteria, and procedures described elsewhere (4). All rats received water and Purina rat chow *ad libitum*, with 3 times weekly supplements of lettuce and celery greens.

Specific procedural details. Litter size. All litters were adjusted to the desired size on day 1 or day 2 postpartum.

Pup growth. The mean daily increment in pup weight was used in some of the experiments dealing with the effect of litter size on FTH secretion. This value was obtained from:

$$\frac{\left[\frac{\text{litter weight at final weighing}}{\text{number of pups}}\right] \text{ minus } \left[\frac{\text{litter weight at initial weighing}}{\text{number of pups}}\right]}{\text{[postpartum day of final weighing] minus [postpartum day of initial weighing]}}$$

In the experiments dealing with the maintenance of lactation in hypophysectomized rats bearing autotransplanted pituitaries, pup growth was compared in the several experimental groups by plotting the mean pup weight per litter against the time after operation.

Vaginal smears. These were obtained with a medicine dropper and distilled water, and read immediately in the fresh state. Some of the rats showed only a single smear of either nucleated or cornified epithelial cells (rather than the usual sequence of nucleated, followed by cornified, cells) at the end of the first postpartum diestrus. For this reason, "estrus," in the term "interval to first estrus," is defined as the day on which the smear first changed from leucocytes to either of the epithelial cell types, regardless of whether the following day's smear was again leucocytic, or of the estrus type.

Gonadotrophin assays. Webster-Swiss white mice were received from Rockland Farms at 21 days of age; the first injection of test material was made on the morning after arrival. The mice were housed in glass jars, with shavings for bottom cover; water and Purina Rat chow were supplied *ad libitum*.

The total administered dose of test material was taken up in a volume of 3.0 cc. of saline per mouse and administered in 0.5 cc. amounts, subcutaneously, twice a day, for 3 days. The mice were killed by breaking their necks on the morning after the last injection; the uteri were removed, cleaned, freed of excess fluid, and weighed on a Roller-Smith torsion balance.

Collection of pituitaries. The pituitaries to be assayed were removed immediately after death, freed of adherent blood on filter paper, and weighed; after discarding the neurohypophysis, the adenohypophysis was crushed between two clean glass slides, and allowed to dry in air. All pituitaries of a given type were collected together on one pair of glass slides, and were stored in a desiccator over calcium chloride except during the collection periods. After storage for at least a week, the dried material was scraped off with a sharp razor blade, ground to a fine powder in an agate mortar and pestle, weighed, and either taken up immediately in saline for injection, or replaced in the desiccator until needed.

FTH preparations. The FTH's tested by the mouse uterine weight method, in combination with LTH, were FSH 125,[2] a purified but relatively weak FSH-like preparation

[2] I am indebted to Dr. W. H. McShan of the Department of Zoology, University of Wisconsin, for the supplies of FSH 125 and F 125, and to Dr. Edward Reifenstein, Jr., E. R. Squibb and Sons, for the supplies of Pituitary Gonadotrophin Squibb, used in this study.

from sheep pituitaries; F 125,[2] a highly potent but unfractioned gonadotrophin preparation from sheep pituitaries, and Squibb's Pituitary Gonadotrophin (SPG),[2] a very potent, unfractionated preparation from horse pituitaries. The two former were available as unsealed powders, which were weighed out and suspended in saline, only as needed; the latter came in a sealed ampule, and was dissolved within the ampule with the attendant diluent. A particular stock solution of SPG, containing 100 R.U./cc., was stored in the refrigerator for no longer than 4 weeks. A diluted stock solution, containing 10.0 R.U./cc. was made from it each week, as needed. There was no noticeable decrease in potency of any of the preparations over the period of time included in the study.

The LTH used for injection (Experiments I, II) was a gift of Prolactin from the Endocrinology Study Section, National Institutes of Health, U.S.P.H.S. It was a highly purified preparation of sheep pituitaries first estimated to contain 20.0 I.U./mg., but later found to contain 15.0 I.U./mg. Each ampule contained 25.0 mg. of dried powder. This was dissolved in 6.25 cc. of saline, and the resultant stock solution (60.0 I.U./cc.) used in the preparation of the final solutions for injection. The total volume of injection fluid was made up from the stock solution of the particular gonadotrophin being tested, the stock Prolactin solution, and sufficient saline to make up the desired volume for each group of mice.

Operations. All operations were done with ether for anesthesia and with clean, but not aseptic, technique. The lactating animals received no special postoperative care except that they were allowed to recover thoroughly from anesthesia before being replaced with their litters. *Ovarian grafts.* Following surgical removal of both ovaries, one of them was kept moist with physiological saline, dissected free of gross luteal tissue, and the remaining stroma cut into 4 pieces, of roughly 1 mm. diameter each. The left kidney was exposed and prepared as for pituitary transplantation (see below), and each piece of ovarian tissue placed beneath the capsule in one of the 4 quadrants on the ventral surface of the kidney. *Hypophysectomy and pituitary autotransplantation.* Hypophysectomies were done by the parapharyngeal approach. The gland was removed by gentle suction, and washed with physiological saline into a trap bottle on the suction line, from which it was immediately removed and placed in a small dish with fresh saline. The left kidney was exposed through a flank incision and a 2 mm. slit made in the midportion of the capsule; through this a small pocket was made with a flattened glass rod on the ventral surface of the kidney between the capsule and parenchyma. The pituitary was inserted into this pocket, the kidney replaced carefully into the abdominal cavity, and the wound closed. No more than three minutes elasped between the recovery of the pituitary and the replacement of the kidney into the abdominal cavity.

Because serial sections of the pituitary regions of the hypophysectomized animals were not made, a description of the means used to evaluate the completeness of hypophysectomy is in order for a proper interpretation of the results. Operations noted to be complete were those in which the gland was recovered from the wash fluid in either one piece (including the neurohypophysis in its normal relation to the rest of the gland), or with a break in the adenohypophysis along its connection with the neurohypophysis. In these cases the latter was either free in the wash fluid, or clinging to one of the halves. These broken glands could be easily reconstituted into the entire gland; in every case so noted, no evidence of remaining tissue could be seen in the pituitary region, at autopsy, even with the aid of dissecting binoculars (×15 magnification). Operations noted to be incomplete were those in which a significant part of one half or more of the adenohypophysis could not be accounted for in the wash fluid, even though inspection through the drill hole showed no evidence of remaining tissue, or those in which the reconstituted parts of the gland showed signs of unusual tears or breakage. No animals of this type were included in the Results.

Autopsies and histologic preparations. The rats were killed with ether; if the autopsy

included removal of the pituitary for gonadotrophic assay, this was done before the rest of the autopsy was started. Usually, the ovaries, ovarian or pituitary grafts, vagina, uterus, adrenals, thyroids, and a portion of each mammary gland or an entire set of mammary glands, were removed, weighed where indicated, and saved in Bouin's fluid. The gross appearance of the ovarian grafts was noted at autopsy, and the entire kidney then placed in Bouin's fluid until the rest of the autopsy was completed. The grafts were then easily peeled off the now somewhat toughened capsule, immediately weighed as a group, and replaced in Bouin's fluid. The fixed tissues were embedded in paraffin, cut at 10 μ, and stained with Harris' hematoxylin and eosin. Further pertinent details of procedure, including the administration of oxytocin (Parke, Davis' Pitocin®) will be described with the Results.

RESULTS AND THEIR INTERPRETATION

The Effect of LTH on the Response of the Ovary to Folliculotrophin Stimulation

Experiment I. The effect of LTH administered together with FTH on mouse uterine weight. One hundred and thirty-three mice were injected with FSH 125, 146 mice with F 125, and 159 mice with Squibb's Pituitary Gonadotrophin (SPG). These gonadotrophins were first administered alone to establish a dose-response curve, and then with various amounts of LTH. The results are shown in Figures 1, 2 and 3. Each curve represents a mini-

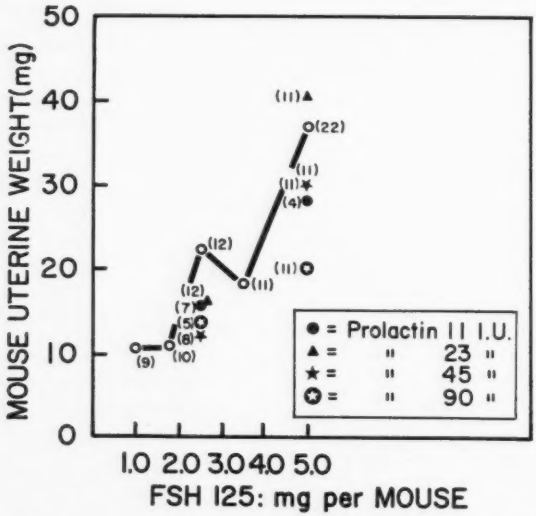

FIG. 1. The effect of prolactin (USPHS) administered together with FSH 125 on the mouse uterine weight response to the latter. FSH 125 was a relatively weak, but pure, FSH from sheep pituitaries (see Footnote 2). Its effect alone is shown by the curve, and in combination with prolactin, by the isolated symbols. The numbers of mice used at each dose are shown in parentheses; those to the left of a given value represent the prolactin-treated ones; the others represent those treated with FSH 125 alone.

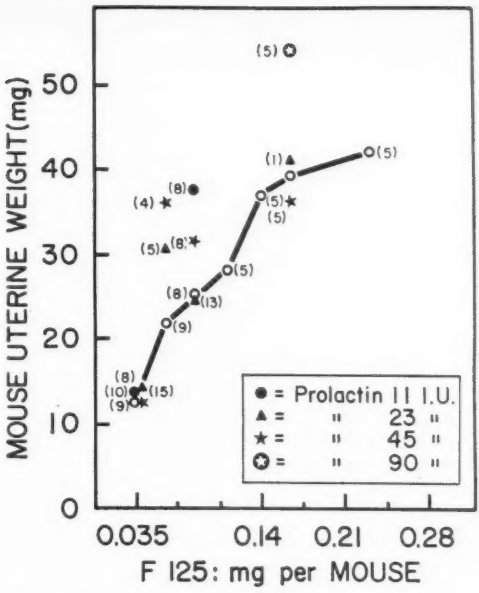

FIG. 2. Same as Figure 1, using F 125, a potent unfractionated gonadotrophin preparation from sheep pituitaries (see Footnote 2).

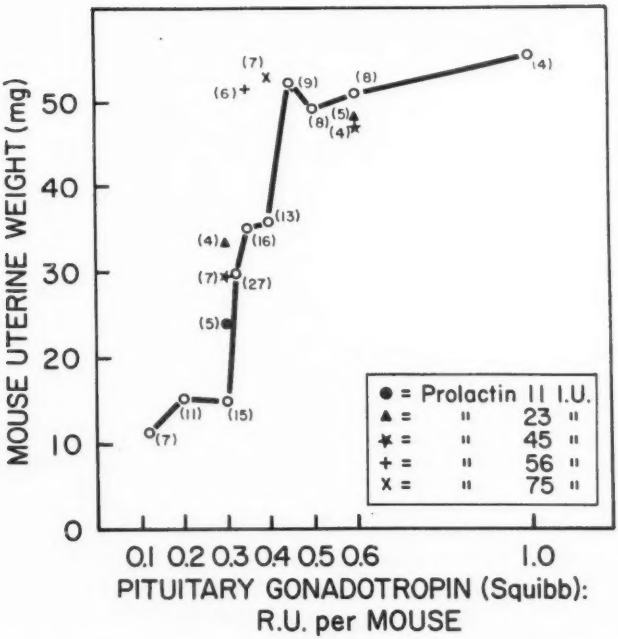

FIG. 3. Same as Figures 1 and 2, using Pituitary Gonadotrophin Squibb (SPG), a highly potent, unfractionated preparation from horse pituitaries (see Footnote 2).

mum of 3 separate runs, and each LTH-FTH combination (with only a few exceptions) the average of at least 2 runs.

The small increase in mouse uterine weight produced with 2.5 mg. of FSH 125 alone was depressed when each of the 4 LTH doses was added. The greater response obtained with 5.0 mg. of FSH 125 alone, however, was appreciably depressed only with the 90 I.U. dose of LTH (Fig. 1). In contrast to this, none of the LTH doses used with F 125, or SPG, had any appreciable effect on the responses (Figs. 2, 3).

Experiment II. The effect of administered LTH on the interval to first estrus in castrated rats bearing ovarian autotransplants. Twenty adult cycling rats were castrated and autotransplanted with ovarian tissue on the day of proestrus, or on day 1 of a diestrus. Ten animals then received each daily injections of LTH (90 I.U./rat), administered in 6 divided doses per day, every 4 hours, and the remaining 10, saline (0.25 cc./dose), in the same way, until the first proestrous or estrous smear appeared. Each group contained equal numbers of animals started at the two different periods of the estrous cycle.

One of the LTH treated rats showed her first estrous smear 6 days postoperatively, the remaining 9, at 7 days. The intervals in the saline treated rats were 3 days in one rat, 6 days in 5, and 7 days in 4. All animals were autopsied on the 8th postoperative day. Nine of the 10 saline-treated rats had typically ballooned uteri, while in the 10th the uterus was thin-walled, but not ballooned. In the LTH treated group, 8 of the animals' uteri were ballooned, one was thin-walled but not ballooned, and one was solid.

The ovarian grafts of the saline-treated rats contained early corpora lutea, or early luteinization of medium and large follicles. In the LTH-treated rats, there seemed to be more prominent thecal luteinization, and fewer early corpora lutea (Fig. 4). Follicular size in both sets of transplants was equal.

Experiment III. The growth of ovaries of cycling, pseudopregnant, and lactating rats in response to exogenous FTH. Twenty-seven lactating, 26 pseudopregnant, and 53 cycling rats were injected with Squibb's Pituitary Gonadotrophin (SPG). Nine lactating, 13 pseudopregnant, and 15 cycling rats served as uninjected controls. SPG was injected over the range of 5 to 25 R.U. in the lactating and the pseudopregnant rats, and over the range of 2 to 32 R.U., total dose/rat in the cycling rats. Injections were started in the cycling rats on day 2 of a diestrus, in the pseudopregnant rat on day 4 of the diestrus, and in the lactating rats on day 10 postpartum. The total dose was administered to each rat in 6 divided doses, given twice a day for 3 days; autopsy took place on the morning after the last injection. Each injection was made in a volume of 0.5 cc. of saline. At autopsy, the ovaries were carefully dissected, and weighed immediately on a Roller-Smith torsion balance.

The mean ovarian weight at each dose of SPG for the 3 groups of rats is

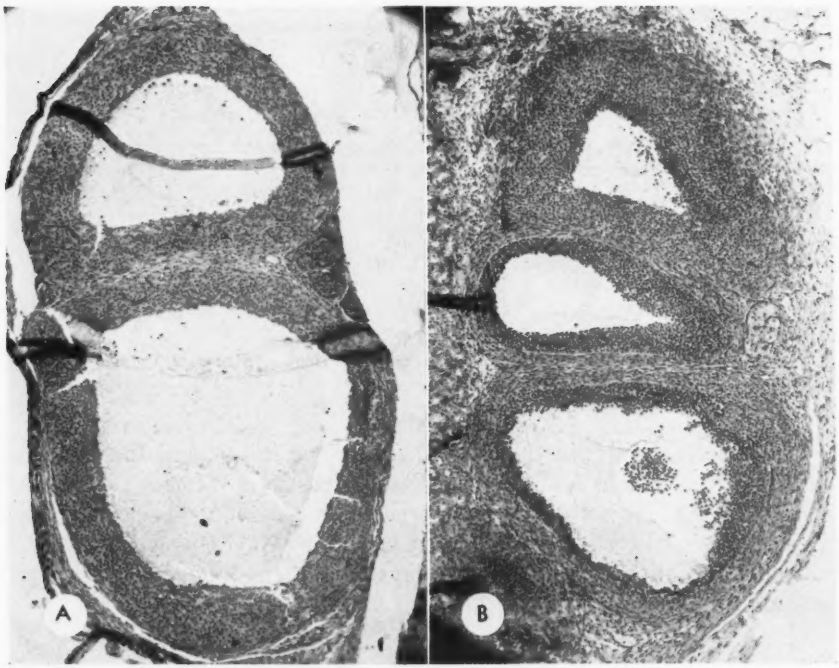

FIG. 4. Low power (×80) photomicrographs of ovarian autotransplants in castrated cyclers; castration and autotransplantation on day 1 of diestrus, and autopsy 8 days postoperatively. A) saline treated control; B) treatment with 90 I.U. prolactin/day from operation to autopsy. Both animals showed a 7 day interval to first postoperative estrus, and ballooned uteri at autopsy. The saline treated animal's graft shows very early luteinization of two large follicles; the prolactin treated one's, a marked degree of thecal luteinization.

shown in Figure 5. The 3 curves were essentially the same, although the somewhat steeper slope of the cyclers' curve at the lower dose levels may have reflected a somewhat greater sensitivity of their ovaries to exogenous folliculotrophins.

The vaginal smears of all SPG injected lactators remained unchanged (leucocytes only) up to and including the day of autopsy. Pup growth was also unaffected (Table 1). No appreciable change from their previous vaginal smear patterns was noted in the injected cycling rats. However, departures from the typical daily leucocytic smear of pseudopregnancy was noted in some of the injected pseudopregnant rats. One of the 6 rats injected with 5 R.U. showed a proestrus-estrus sequence on days 6 and 7 (day 3 of injection and day of autopsy); 3 of the 7 rats injected with 10 R.U. showed an isolated proestrous or estrous smear on day 6; similar smears were seen in 3 rats on day 6, in the group injected with 15 R.U., while two more in this group showed a proestrus-estrus sequence on days 5 and 6, and

TABLE 1. THE EFFECT OF TREATMENT OF INTACT LACTATING RATS WITH PITUITARY GONADOTROPHIN (SQUIBB) ON THE GROWTH OF THEIR LITTERS (FOR DETAILS REGARDING PROCEDURES SEE RESULTS; EXPERIMENT III)

	Total dose SPG/rat (R.U.)				
	0	5	10	15	25
Number of rats	9	6	7	7	7
Litter size					
Mean (No. pups)	7.7	8.2	8.3	8.0	8.3
Range (No. pups)	4–11	6–10	7–10	6–10	7–9
Pup weight on day 14 post-partum					
Mean/litter (gm.)	24.9	26.6	25.7	25.1	24.7
Range of mean/litter (gm.)	21.5–28.4	23.4–28.7	19.0–30.3	21.9–33.3	23.6–26.3

6 and 7, respectively; in the 25 R.U. group, one rat showed the latter sequence on days 6 and 7, and 3 the isolated proestrous or estrous smear pattern on day 6. This frequency of proestrous or estrous smears is much greater than expected in our colony at any time earlier than the 9th day in animals that have gone through 4 days of diestrus following mechanical stimulation of the cervix.

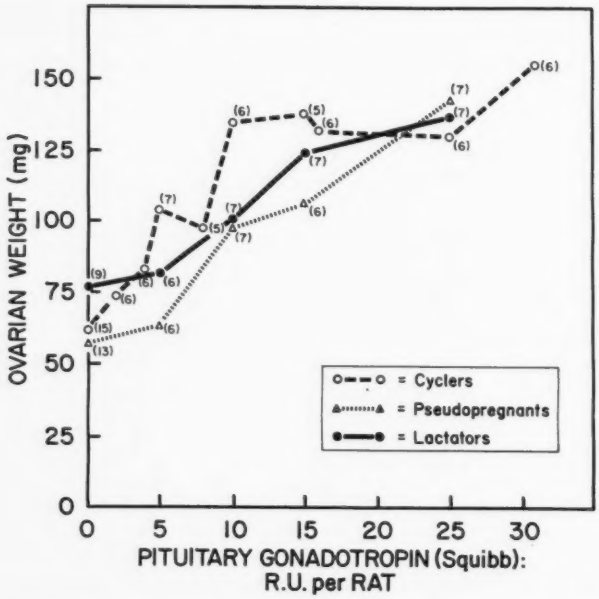

FIG. 5. The response of the rat ovary to injected gonadotrophin (Pituitary Gonadotrophin Squibb) (see Footnote 2); cyclers, pseudopregnants and lactators compared. The numbers of animals at each dose are shown by the numbers in parentheses.

TABLE 2. DISTRIBUTION OF THE VARIOUS LITTER SIZES OF INTACT MOTHERS, AND THEIR SURVIVAL (EXPERIMENT IV)

Litter size (No. of pups)	Total number of mothers	Surviving litters	
		No.	%
1	54	21	38.9
2	19	14	73.7
3	17	8	47.1
4	16	11	68.8
5	2	1	50.0
6	1	1	100.0
7	5	4	80.0
8	2	2	100.0
9	5	5	100.0
10	3	2	66.7
12	1	1	100.0
5 or more	19	16	84.2

Interpretation of Experiments I, II, and III. The three experiments demonstrate that neither LTH, nor the conditions of its secretion, account for follicular quiescence. They therefore validate the *signs* of FTH activity in the following experiments, as measures of *actual* FTH secretion, or its absence. The increased frequency of estrus-like smears in the SPG-treated pseudopregnant rats will be considered in the General Discussion.

The Relation between Litter Size and Folliculotrophin Secretion

Experiment IV. The interval from delivery to the first vaginal estrus in intact lactating mothers. Of the 125 mothers with which this experiment started, 70 maintained their litters until weaning, with only partial losses within a few litters. Over 50% of the 1-pup litters failed to survive; survival rates of litters of 2 pups or more, however, were probably not significantly different from one another (Table 2).

The intervals from delivery to the first vaginal estrus (disregarding that associated with delivery) are shown in Figure 6. This graph also includes data on 50 control mothers, whose litters died or were removed in toto on day 1 or 2 postpartum. There was a definite tendency for the interval to the first postpartum estrous smear to increase with increase in the size of the litter. With the exception of the control group, the intervals within each litter-size group were distributed unimodally, skewed somewhat to the left. This was most pronounced in the groups with 1- and 2-pup litters, but was still faintly apparent in those with larger litters. The distribution of intervals in the controls was definitely bimodal, the two modes being 5.5 and 13 days, respectively (Fig. 6, 0 pups). Any particular control's interval was apparently unrelated to how her litter was reduced to zero, since there were approximately equal numbers of litters that died, or were removed, in each of the two subgroups.

If we consider only the controls' higher mean interval to first estrus as a

starting point, both the means and modes of the intervals appear to increase in straight line fashion with litter size (Fig. 6).

Vaginal smears were discontinued in some mothers at weaning, and weaning took place, in some cases, as early as day 19 postpartum. Where weaning was delayed, and vaginal smears were continued, the incidence of pseudopregnancy-like diestrus periods (9 to 17 days) with continued suckling after the first postpartum estrus could be observed. Of 19 such mothers with 1-pup or 2-pup litters, 15 showed such diestrus periods after the first postpartum estrus, and one more, after the second postpartum estrus. Of the remaining 21 suitable mothers, the interval to first estrus was over 21

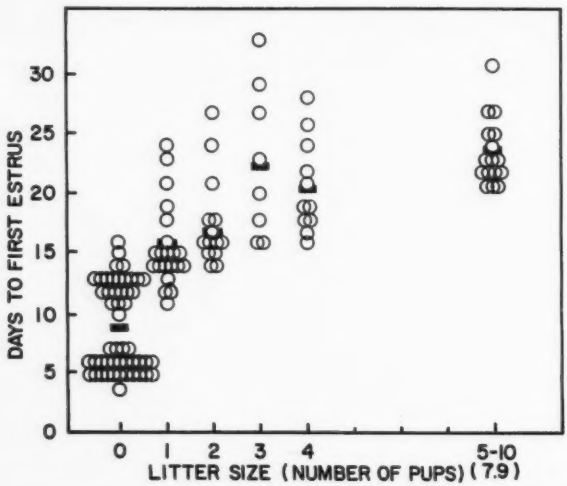

FIG. 6. The relation between litter size and the interval to first vaginal estrus postpartum in intact rats. Individual values are shown by the open circles, means for the group by the solid black bar. The sizes of the litters in the 5–10 pup group are shown in Table 1. For further details see text (Experiment IV).

days in 14; none of these mothers showed subsequent pseudopregnancy-like diestrus periods. Of the 7 with intervals to first estrus of 15 to 21 days, however, 4 showed such diestrus periods.

Interpretation of Experiment IV. The increase in duration of the postpartum diestrus with increase in litter size could be due to a progressively greater suppression of FTH secretion and/or a progressively greater secretion of LTH. The former possibility was tested in the next three experiments. The latter could not be tested directly. The secretion of LTH in amounts at least equivalent to those in pseudopregnancy by *all* nursing mothers, however, was demonstrated by the immediately following evidence, plus that presented in Experiment VIII (see also General Discussion).

The change in the incidence of pseudopregnancy-like intervals to first estrus from slightly less than 50% in the controls, to 100% in the mothers with 1-pup litters suggests that minimal suckling was enough to stimulate LTH secretion in *all* rats. The high pituitary FTH potential of the castrated mothers with small litters (Experiments V, VI, VII) shows that this change was not due to FTH suppression, and therefore establishes it as due to the action of LTH on the corpora lutea, as in pseudopregnancy. LTH secretion, therefore, cannot be a spontaneously continuous process, but one that results from specific stimuli. The latter conclusion is also supported by the findings of Mühlbock and Boot (5), and Quilligan and Rothchild (6),

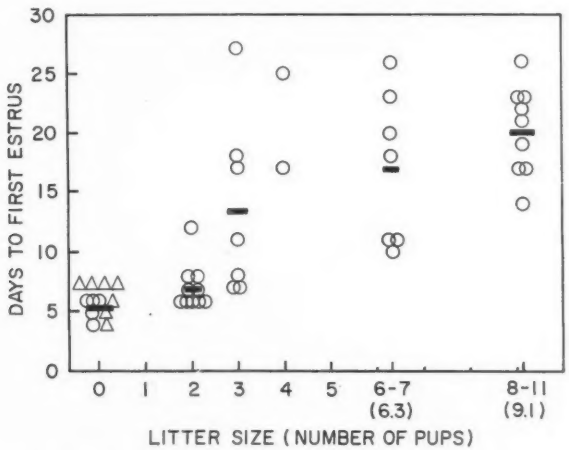

FIG. 7. The relation between litter size and the interval to first estrus postoperatively, in castrated lactating rats bearing ovarian autotransplants. The control group consisted of 5 rats whose litters were removed entirely at operation, and 7 rats, whose litters died before day 7 postpartum, with no evidence of milk ingestion. The latter are shown by the triangles.

and by the length of the diestrus following the first postpartum estrus. This tendency for estrus to recur at intervals characteristic of pseudopregnancy was also observed by Hain in rats nursing small litters (7), and by Selye and McKeown in nulliparous rats and mice that suckled adopted litters (8).

The further significance of this experiment, which confirms the earlier reports of Hain (7), in rats, and of Parkes (9), and Parkes and Bellerby (10), in mice, will be considered in the General Discussion.

Experiment V. The interval to first vaginal estrus in castrated lactators bearing autotransplanted ovarian tissue. Forty-seven mothers were castrated, autotransplanted with ovarian tissue, and their litters adjusted to the desired size on day 2 postpartum. The individual and mean intervals to the first postoperative vaginal estrus are shown in Figure 7.

These rats also showed a direct relationship between litter-size and interval to first estrus. However, *all* the controls' intervals were remarkably close to the lower of the two modal values seen in the intact controls. The intervals in all groups were definitely shorter than they were in the intact mothers with litters of equivalent size. The difference in mean interval between the intact mothers and those bearing ovarian autografts was of the order of 7 to 9 days when no litter, or a small one was suckled, and about 5 days, when a larger litter was suckled (Table 3).

The postoperative observation period was long enough, and the interval to first estrus short enough in 34 rats, for adequate observation of the incidence of "spontaneous" pseudopregnancies following the first postopera-

TABLE 3. THE MEAN INTERVAL TO FIRST ESTRUS IN RELATION TO LITTER SIZE; INTACT LACTATORS COMPARED WITH CASTRATED LACTATORS BEARING OVARIAN AUTOGRAFTS (EXPERIMENTS IV AND V)

Litter size[1] (No. of pups)	Intact lactators		Lactators with ovarian autografts		Difference in mean interval (days)
	Number of rats	Mean interval (days)	Number of rats	Mean interval (days)	
0	23[2]	12.7[2]	12	5.8	*6.9*
2	14	16.5	10	7.2	*9.3*
3	8	22.8	7	13.6	*9.2*
5–11 (mean: 7.9)	16	23.7	16	18.8	*4.9*

[1] The group of castrated lactators bearing ovarian autografts did not include any 1-pup litters, and the number of 4-pup litters was too small for comparison. The numbers of mothers within each of the litter size groups of 5 pups or more were too small for comparison, and for this reason the "large" litters were compared as a group.

[2] This is only the group showing the prolonged interval to first estrus; the other 27 rats in this control group had a mean interval to first estrus of 5.7 days (see Figure 6, and text)

tive estrus. Of the 12 controls (no litters, or incompletely maintained litters; Fig. 7), only one showed such a pseudopregnancy. Of 16 suitable mothers with 2- or 3-pup litters, 8 showed 9 to 16 day diestrus periods following the first postoperative estrus, and 2 more showed diestrus periods of 9 to 12 days after the 2nd postoperative estrus. Four of the 5 suitable mothers with large litters (6 pups or more) showed 9 to 16 day diestrus periods, following the first postoperative estrus.

Experiment VI. The growth of ovarian autotransplants in castrated lactators. The weights of the ovarian grafts of the animals just described were plotted against the postoperative interval to autopsy,[3] and in relation to

[3] The intervals to autopsy and first estrus did not usually correspond except for some of the rats with the larger litters. The reason for this was that in order to kill all the rats at approximately the same time from operation, we attempted not only to keep the mothers with the shorter intervals alive for periods roughly equal to the longer intervals to first estrus in the rats with larger litters, but we also tried to observe their intervals to second estrus. This caused a certain amount of unavoidable variation, but even so, the mean periods between operation and autopsy of the controls, and of mothers with 2, 3 and 6 or more pups were not very different (25.4, 24.0, 23.9 and 23.3 days, respectively).

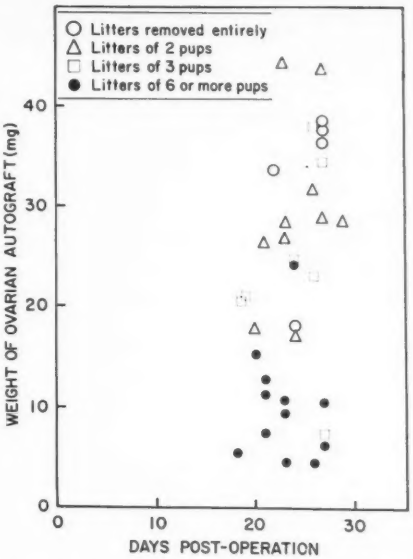

FIG. 8. The relation between litter size and the weight of the ovarian transplants, in castrated lactators bearing ovarian autotransplants. The data are plotted against the postoperative interval to autopsy. (See Footnote 3 for explanation of the variation in the interval to autopsy. The two 4-pup litter values were not included in the graph, in order to keep it as simple as possible; their grafts weighed 23.8 and 35.4 mg., respectively.) Four of the mothers with litters of 6 pups or more were killed about a month later than any of those included in the figure, and after their litters were weaned. Their grafts were obviously not comparable to those shown here, and for this reason were excluded.

litter size (Fig. 8). The size of the transplants seemed to be inversely related to litter size. This inverse relationship is shown more dramatically by Figure 9, in which the minor differences between the various groups' intervals to autopsy have been omitted.

The weight of each mother's ovarian graft was also plotted against her interval to first estrus, without regard to litter size or interval to autopsy; these data also showed an inverse relationship between graft size and interval to first estrus (Fig. 10).

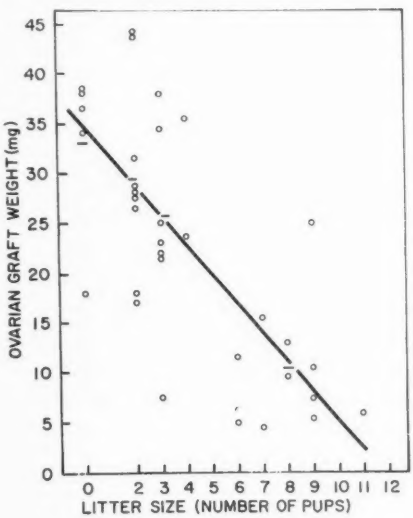

FIG. 9. The relation between litter size and the weight of the ovarian transplants in castrated lactators bearing ovarian autotransplants. There are the same data shown in Figure 8 plotted without regard to the intervals from castration to autopsy. The values for the 3- and 4-pup litters were combined to give the mean shown, as were those for all litters of 6 or more. (See text (Experiment VI) for further details.)

At autopsy, the controls' grafts and the grafts of the mothers of the small litters were almost always strikingly different from those of the mothers of the larger litters. The latters' grafts were small and flat, and consisted mostly of follicles; corpora lutea, when present, were few, and relatively fresh looking. In contrast, the transplants in the former animals appeared to be packed with corpora lutea of several sizes, and though follicles were present, they did not usually make up the bulk of the ovarian tissue. These gross differences were confirmed by microscopic examination of the transplants (Fig. 11).

At the time of autotransplantation, the 4 grafts together weighed

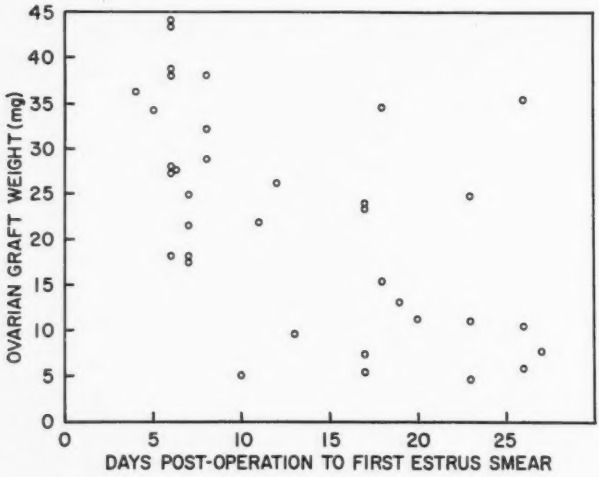

FIG. 10. The relation between the interval to first estrus postoperatively, and the weight of the ovarian transplants in castrated lactators bearing autotransplanted ovarian tissue. Litter size and the interval to autopsy have been excluded; the inverse correlation has not been tested for statistical significance, but is fairly obviously present.

roughly 10.0 mg. in each rat. It can be seen from Figure 9 that this is very close to the average weight of the transplants removed at autopsy in the mothers with large litters. It has been our experience, from other experiments, that these grafts largely atrophy immediately after transplantation, and then begin to grow. The differences in size between the grafts of the mothers with large litters, and those with small or no litters, therefore, probably represent differences in rates of true growth, rather than between stasis on the one hand, and growth on the other.

Experiment VII. The FTH potency of the castrated lactator's pituitary in relation to litter size. The pituitaries of intact lactating rats with full litters (5 pups or more), of castrated lactating rats with full litters, or with 2-pup litters, and of castrated post-parturient rats whose litters were removed en-

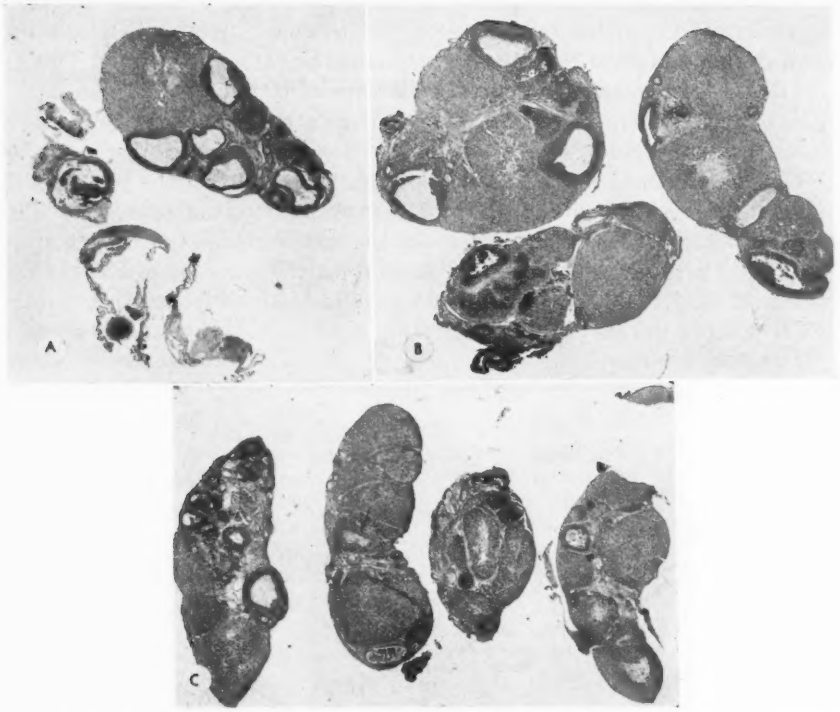

FIG. 11. Low power (×14) photomicrograph of the ovarian autografts in castrated post-parturients nursing no pups, 2 pups, or full litters. A) *Full litter* (6 pups; interval to first estrus was 20 days, and grafts weighed 11.4 mg.); B) *2-pup litter* (interval to first estrus was 6 days, and grafts weighed 27.6 mg.); and C) *no litter* (interval to first estrus was 6 days, and grafts weighed 36.6 mg.). B and C show many corpora lutea of different ages; the corpus luteum in A was the only one seen in all 4 grafts; the other 3 grafts in this animal were considerably smaller than the one containing the corpus luteum. (The apparently larger size of the grafts in B than in C is deceiving, since the latter actually covered a larger area of kidney than the former.)

tirely, were collected and assayed by the mouse uterine weight method. In all cases, castration and litter reduction or removal was done on day 2 postpartum, and autopsy on day 16 postpartum. These assays (Fig. 12) demonstrated that: a) in the rats nursing a full litter, castration did not result in any detectable increase in pituitary FTH potency, while removal of the litter was associated with a marked increase in FTH potency following castration, and b) the response to castration of mothers nursing 2-pup litters was indistinguishable from that of mothers whose litters had been removed entirely.

Interpretation of Experiments V, VI, and VII. These experiments demonstrate that FTH secretion (formation and release) was suppressed during

lactation in proportion to litter size. The demonstration of FTH suppression during lactation confirms and extends the earlier reports by Desclin (11), and Desclin and Gregoire (12). Assays of pituitaries and plasma for LH, by means of the ascorbic acid depletion method, have so far yielded results that parallel these to a remarkable degree (13). The suppression of FTH secretion was not mediated through the effects on the pituitary of LTH itself, nor through those of progesterone secreted in response to LTH, for the following reasons. LTH does not prevent the formation of castration cells in rat pituitaries (14). LTH did not affect the secretion of FTH in Experiment II. LTH does not affect the post-castration increase in pituitary FTH potency in rats (15). The lactation diestrus continues in the absence

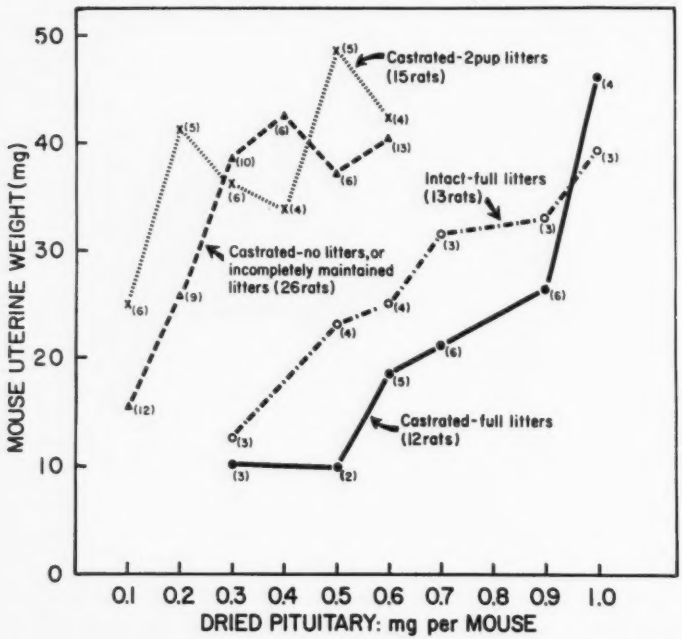
MOUSE UTERINE WEIGHT (mg)
DRIED PITUITARY: mg per MOUSE
Castrated-2pup litters (15rats)
Intact-full litters (13rats)
Castrated-no litters, or incompletely maintained litters (26rats)
Castrated-full litters (12rats)

FIG. 12. The effect of litter size on the folliculotrophic potency of the lactating rat's pituitary, tested by the mouse uterine weight method. The small numbers shown in parentheses near each mean value represent the number of mice used at the particular dose level. Each assay was completed in at least 2 runs, using at least 3 doses in each run. The 26 rats included in the group of castrates with no litters or with incompletely maintained litters consisted of 10 rats whose pups were removed on day 2 postpartum, and 16 that failed to maintain their litters beyond the 5th postoperative day. The pituitaries of these two groups were so similar in potency that the assay results were combined to give the average curve shown. Both this and the 2-pup litter curve are in the same range we have repeatedly found for castrated cyclers, two weeks postoperatively. The two lower curves are in the same range as intact cyclers. (See text (Experiment VII) for further details.)

of the corpora lutea (16) (and Experiment V; corpora lutea atrophy rapidly in these transplants (17)). Progesterone does not depress the FTH potency of either intact or castrated rats' pituitaries (15).

The FTH suppressing effect of litter size was unrelated either to the amount of milk secreted (see Experiment VIII), or to inanition; with only a few exceptions, *all* mothers gained weight during the course of lactation. Suckling itself, therefore, must be the causative agent in the effect of litter size on FTH secretion, and its effect must very probably be exerted on the processes that control FTH secretion.

The length of the interval to first estrus in the castrated mothers bearing ovarian autotransplants (Fig. 7) was thus determined practically entirely by the extent to which suckling suppressed FTH secretion. In the intact mothers (Fig. 6) this effect was still the primary determinant of the *variation* in length of the diestrus, but the actual *duration* of the interval was also affected by the influence of progesterone on FTH release from the pituitary (Table 3; see also the General Discussion).

The Effect of Suckling on the Secretion of Luteotrophin

Experiment VIII. Pup growth as an index of LTH secretion: its relation to litter size, and to the signs of FTH secretion. The mean daily increments in pup weight of the litters of the intact and of the castrated mothers of Experiments IV–VI were plotted against litter size (Fig. 13). Pup growth in the intact mothers' small litters was variable, particularly in the 1- and 2-pup litters, but included many values within what may be considered the normal range, i.e., that shown by the larger litters of the intact mothers. With only 4 exceptions, the growth of the 3- and 4-pup litters, however, was well within this range. There was practically no relationship between litter size and pup growth in the litters of the castrated mothers. Pup growth in

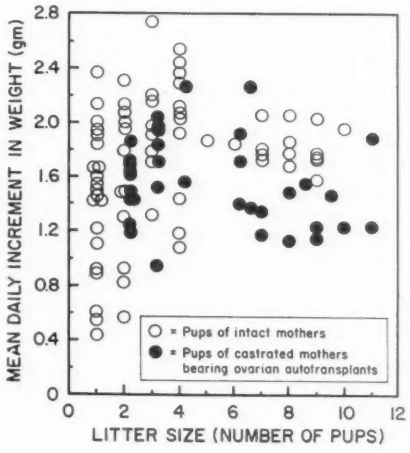

FIG. 13. The relationship between litter size and the mean daily increment in pup weight in the litters of intact mothers, and of castrated mothers bearing ovarian autografts. (See text (Experiment VIII) for further details.)

the larger litters of these mothers, however, was definitely less than in those of the intact mothers (Fig. 13). Pup growth was compared in the same way in the litters of the mothers of Experiment VII, and is shown in Table 4; these data show the same lack of relationship between pup growth and litter size that is seen in Fig. 13.

Since there were obviously fewer evidences of suppression of FTH secretion in the mothers of the smaller litters, the data were re-examined to see whether poor pup growth was related to the mothers' increased signs of FTH secretion. The interval to first estrus was chosen as the index of FTH secretion. Each group of intact mothers nursing a litter of a particular size was divided into two groups, one of which showed "short," and the other "long" intervals to first estrus. The dividing point between short and long intervals in each group was selected to correspond as closely as possible to

TABLE 4. PUP GROWTH IN THE LITTERS OF INTACT MOTHERS, AND OF CASTRATED MOTHERS WITH FULL LITTERS (5 OR MORE), AND IN 2-PUP LITTERS OF CASTRATED MOTHERS[1]

		Intacts with full litters	Castrates with full litters	Castrates with 2-pup litters
Mean daily increment in pup weight[2]	No. of litters	13	12	15
	Mean of the group (gm.)	1.95	2.15	1.95
	S.D. (gm.)	0.14	0.17	0.31
	Range (gm.)	1.71–2.26	1.86–2.39	1.18–2.46

[1] See Results; Experiment VII.
[2] From day 2 to day 16 postpartum.

the mean for the group. Within each subgroup, then, the mean and standard deviation of the pups' mean daily increment in weight was calculated. The same procedure was used for the litters of the castrated mothers bearing ovarian autografts, except that the data were considered in only two litter-size groups. The data (Table 5) demonstrate the lack of a consistent relationship between pup growth and the mothers' intervals to first estrus. A spot check of the relationship between pup growth and the size of the ovarian grafts in the castrated mothers showed a similar lack of correlation.

Interpretation of Experiment VIII. These data and those of Experiment IV demonstrate that the mothers of *small* litters secreted LTH in amounts at least equivalent to those in pseudopregnancy. These mothers simultaneously secreted FTH at rates not far below those of castrates without litters (Experiments V, VI, VII), and therefore, at rates higher than those in intact rats. It therefore follows that LTH secretion cannot be caused by or be dependent on the suppression of FTH secretion.

That FTH suppression was mediated by suckling rather than through the effects of milk secretion itself is suggested by the lack of correlation between pup weight and signs of FTH secretion (Tables 4 and 5). (This is also seen in the fact that roughly equivalent total weights of pups were

TABLE 5. THE RELATIONSHIP BETWEEN THE INTERVAL TO FIRST ESTRUS IN INTACT LACTATING RATS, AND IN CASTRATED LACTATORS BEARING OVARIAN AUTOTRANSPLANTS, AND THE GROWTH OF THEIR PUPS (EXPERIMENT VIII)

Experimental group	Means of the intervals to first estrus and of the pups' mean daily increment in weight					
	"Short" intervals			"Long" intervals		
	No. of rats	Interval to first estrus (days)	Mean daily increment in pup weight (gm.)	No. of rats	Interval to first estrus (days)	Mean daily increment in pup weight (gm.)
Intacts						
One-pup litters: (dividing[1] point = 15 days or less)	15	13.8 (1.3)	1.53 (0.58)[4]	6	20.2 (3.1)	1.28 (0.55)
Two-pup litters: (dividing point = 16 days or less)	8	15.3 (0.9)	1.61 (0.52)	6	20.8 (3.9)	1.60 (0.60)
Three-pup litters: (dividing point = 20 days or less)	4	17.5 (1.9)	2.15 (0.42)	4	28.0 (4.2)	1.80 (0.38)
Four-pup litters: (dividing point = 20 days or less)	6	17.8 (1.2)	2.04 (0.35)	5	24.2 (2.9)	1.85 (0.68)
Five-pup or larger litters:[2] (dividing point = 23 days or less)	10	22.0 (1.3)	1.82 (0.13)	6	26.5 (2.5)	1.86 (0.16)
Castrates with autotransplanted ovarian tissue						
Two- to four-pup litters:[3] (dividing point = 10 days or less)	12	6.8 (0.9)	1.60 (0.26)	7	18.1 (6.1)	1.68 (0.48)
Six-pup or larger litters: (dividing point = 20 days or less)	8	14.6 (3.6)	1.51 (0.39)	8	23.0 (2.1)	1.44 (0.26)

[1] See text for explanation (Results; Experiment VIII).
[2] The average number of pups/litter in each of the two groups shown was 8 and 7.9 respectively.
[3] The average number of pups/litter in the group with the shorter intervals was 2.3; it was 3.1 in the group with the longer intervals.
[4] Standard deviations are shown in parentheses.

raised by some mothers with small heavy litters, and some with large light litters, but there was a greater suppression of FTH secretion in the latter).

Experiment IX. The maintenance of lactation in hypophysectomized lactators, bearing pituitaries autotransplanted to the kidney.

A. *Hypophysectomized lactators bearing autotransplanted pituitaries, without further treatment.* Nineteen mothers were operated on, 12 between the 1st and the 4th, and the remainder on the 5th, 6th, or 7th postpartum day. No pups survived beyond the 6th postoperative day. Although the pups, unless moribund, were almost always found hanging to their mothers' nipples, all pups lost weight, and failed to ingest milk, following operation.

B. *Hypophysectomized lactators bearing autotransplanted pituitaries, treated with oxytocin.* Two pilot experiments were run with twice/day (3 rats), or 4 times/day (4 rats) injections of oxytocin (total dose: 2.0 I.U./day). The mothers were operated on, as in A, between days 4 and 6 postpartum, and injections started on the day following operation. Part of one litter in each of the two treatment groups (3 out of 8 pups, and 8 out of 9 pups, respectively) survived to weaning. The other pups lost weight steadily, in spite of occasional milk ingestion, and died between days 5 and 7 postoperatively. The survivors' weight gains are shown in Figure 14, C, D.

Oxytocin (0.5 I.U., in 0.25 cc. of saline) was then administered every 4

hours "round the clock," from the day of operation (day 5 post partum) until weaning (day 21 postpartum), to 8 mothers operated on similarly. Five litters survived without losses (5 to 9 pups each); 7 out of 12 pups, 5 out of 8 pups and 2 out of 9 pups in the remaining 3 litters survived. All survivors ingested at least some milk from the time of operation forward, but did not begin to gain weight until 2 to 3 days after operation (Fig. 14, A). The relationship between oxytocin injection and milk ingestion was striking; just prior to an injection, the pups' milk spots were almost always absent or small, but an hour later, filled in the entire stomach outline.

These 8 mothers were autopsied at the time of weaning. Their mammary glands were grossly similar to those of the intact controls (control 1, below);

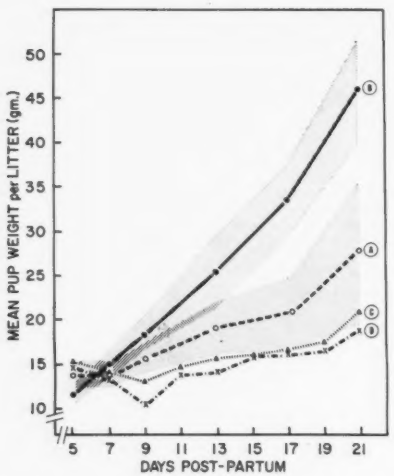

FIG. 14. Average growth curves of the litters of intact lactators and of hypophysectomized rats bearing autotransplanted pituitaries treated with oxytocin. Shaded areas are the standard deviations. A) *Pituitary-autotransplanted lactators*, 8 litters; treated with 0.5 I.U. oxytocin every 4 hours from operation (day 5 postpartum) to weaning; B) *intact lactators* (9 litters); C) *like A*, but given 0.5 I.U. oxytocin 4 times a day, starting on the day after operation (1 surviving litter out of 4); D) *like A*, but given 1.0 I.U. oxytocin twice a day, starting on the day after operation (1 surviving litter out of 3).

that is, they were thick, white, well extended, and vascular, and their cut surfaces exuded milk. A brief survey of representative histologic sections showed that between 50% and 80% of each gland showed active secretion. These proportions overlapped those found in the controls, but some of the latter showed a higher percentage of tissue in active secretion (Fig. 15).

Controls. All Control experiments were done within 2 to 3 weeks of the above experiments. 1) Intact controls were 9 mothers in good lactation on day 5 postpartum. Seven litters (4 to 12 pups each) survived without losses; 4 out of 5 pups and 3 out of 12 pups in the remaining 2 litters survived. Their average weight curve is shown in Figure 14, B.

2) Sham hypophysectomy and laparotomy was done on 7 mothers on days 5 or 6 postpartum. Five litters survived, 4 (7 to 12 pups each) without losses, and 1 (10 pups) with a loss of 4 pups; the remaining 2 litters died by the 6th postoperative day. Their average weight curve is compared with the intact controls and the experimentals in Figure 16.

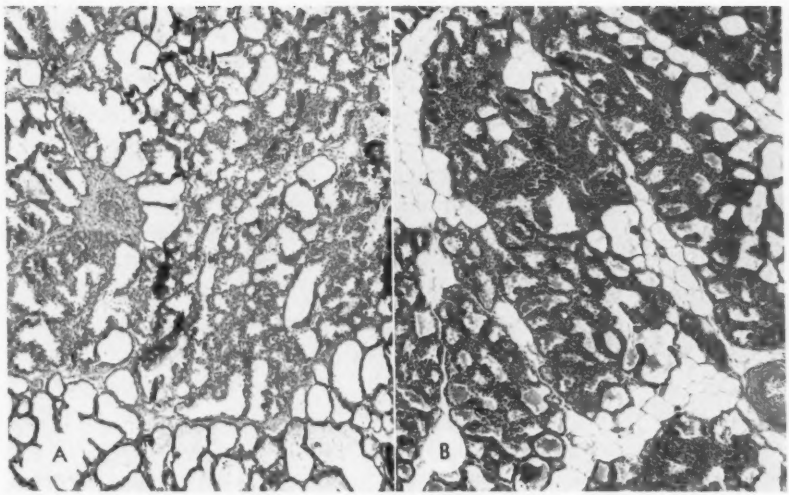

FIG. 15. Low power (×80) photomicrographs of sections through the mammary glands of an intact lactator, and of a hypophysectomized lactator bearing an autotransplanted pituitary treated with 0.5 I.U. oxytocin every 4 hours from operation (day 5 postpartum) to weaning. Autopsy in both cases was on day 22 postpartum. A) *Intact control;* this mother had a litter of 10 pups, whose average weight on day 21 postpartum was 45.3 gm.; B) *Hypophysectomized, pituitary-autotransplanted, and treated with oxytocin;* this mother had a litter of 5 pups, whose average weight on day 21 postpartum was 44.6 gm. The alveoli in both sections are actively secreting, but a larger proportion of the total mammary gland in B is involved in fatty change.

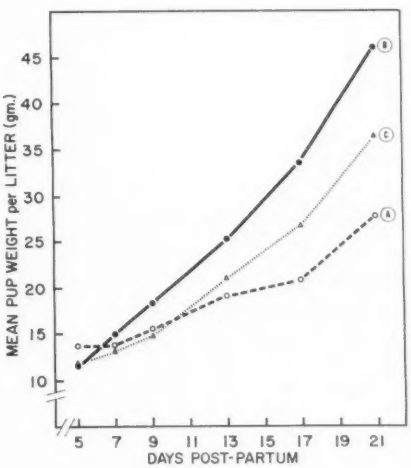

FIG. 16. Average growth curves of the litters of intact lactators, of hypophysectomized rats bearing autotransplanted pituitaries treated with oxytocin, and of sham-hypophysectomized, laparotomized controls. *A) and B) are the same as in Figure 14 with the standard deviations omitted; C) sham-operated controls* (5 litters); the standard deviations have been omitted only for simplicity's sake; they were intermediate between those of A and B in Figure 14.

3, a) 3 mothers were hypophysectomized on day 5 postpartum, and given oxytocin every 4 hours around the clock; b) 6 mothers were similarly hypophysectomized, but not given oxytocin; c) 5 litters were separated from their mothers on day 5 postpartum; and d) 14 mothers were hypophysectomized and pituitary autotransplanted on day 5 postpartum and injected subcutaneously with oxytocin tannate[4] once/day (1.0 to 3.0 I.U./day in either oil or saline). Pup survival and weight loss in these 4 control groups were essentially the same as in the litters of the untreated hypophysectomized mothers bearing autotransplanted pituitaries (A, above). Some of the pups of the mothers treated with oxytocin tannate, however, showed occasional milk ingestion.

Additional pertinent findings. The average adrenal weight of the oxytocin treated experimental mothers (B, above) was 40.5 mg. (32.8–46.6 mg.). In our colony, the average adrenal weight of intact lactators of equivalent body weight is about 65.0 mg., and individual weights of less than 50.0 mg. have never been encountered. The other hypophysectomized animals were killed at later intervals, and showed even more adrenal atrophy, regardless of whether they were pituitary-autotransplanted. The ovaries of all the pituitary-autotransplanted rats had the large, bright pink corpora lutea, and pale yellow sparse stroma that is characteristic of the completely hypophysectomized rat bearing a pituitary transplant (2, 3). A totally unexpected finding was vaginal mucification in the oxytocin treated experimental rats (B, above); the significance of this has been considered elsewhere (18).

Interpretation of Experiment IX. The data demonstrate that milk secretion can occur in response to LTH secreted by a pituitary separated from the CNS. Milk secretion was undoubtedly aided by the direct effect of oxytocin on the mammary gland. The results offer no evidence that oxytocin also affected the rate of LTH secretion, but other evidence that it cannot do so is offered elsewhere (18). It could still be argued that in this experiment oxytocin might have affected the transplant's rate of LTH secretion, perhaps as a pharmacological agent might have done. Other findings, however, suggest that the amount of LTH required for luteal maintenance and for milk secretion are the same, and that amounts of LTH greater than the minimal effective one produce no further increase in milk secretion (19). This implies that since the autotransplanted pituitary can maintain the corpora lutea, it also secretes enough LTH for milk secretion, so that even if oxytocin could affect its rate of LTH secretion, the amount of milk produced would not be increased. The following also suggests that the amount

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[4] Oxytocin tannate was prepared in our laboratories from a solution of concentrated oxytocin donated through the kindness of Dr. D. McGinty, of Parke, Davis and Co. The procedure was the same as that used for the preparation of Parke, Davis' pitressin tannate.

of LTH secreted by the transplant is not small. In parabiotic adult rats, cervical stimulation of one partner during proestrus or estrus induces pseudopregnancy only in the stimulated partner; homotransplantation of a single rat pituitary, however, to one partner, *without* cervical stimulation, induces pseudopregnancy in both (20).

Experiment IX demonstrates then that the secretion of LTH does not depend on stimulation from the CNS. This suggests, therefore, that the effect of suckling is more probably one of "permitting" LTH to be secreted, than one of directly stimulating its secretion.

Two principal factors may account for the below average weights of the pups of the experimental rats: a) oxytocin injection every 4 hours was probably a less efficient means of facilitation of milk withdrawal than that which occurs normally, and b) hormones other than LTH are required for optimal milk secretion (21); the autotransplanted pituitary secretes some of these at rates that are probably less than those in intact animals (2, 3).

GENERAL DISCUSSION

Suckling as the cause of LTH secretion, and of the suppression of FTH secretion. The results and interpretations of these experiments have eliminated all explanations for the association between LTH secretion and follicular quiescence except the one that the conditions that promote LTH secretion also cause follicular quiescence. The following postulations may explain how follicular quiescence during lactation is a consequence of the general process through which LTH secretion is promoted:

1) the pituitary secretes FTH in response to stimuli arising in the CNS; when these stimuli are suppressed, the pituitary cannot secrete FTH.

2) LTH secretion is chronically inhibited by another CNS influence; when its activity is suppressed, the pituitary secretes LTH.

3) suckling suppresses the activity of both influences, and thus permits LTH to be secreted, and depresses or prevents the secretion of FTH.

4) the CNS influence that stimulates FTH secretion is less sensitive to the suppressing effect of suckling than is the one that inhibits LTH secretion.

Let us consider first the available evidence for these postulations. We will then restate the principle underlying them in order to explain the relationship between LTH secretion and follicular quiescence in pseudopregnancy as well.

Evidence for a CNS influence that stimulates FTH secretion. As a result of the finding that transplanted pituitaries in castrated rats did not form castration cells, Hohlweg and Junkmann (22) postulated the existence of a "Nervencentrum" having this role. Certain hypothalamic lesions in rats (23) and dogs (24) abolish the increase in pituitary FTH secretion that follows castration. Minute amounts of estrogen liberated into the hypothalamus of intact rats lead to ovarian atrophy, but have no detectable

systemic effects (25). The pituitary in these experiments showed no signs of injury. The rat pituitary transplanted beneath the median eminence secretes FTH (26, 27) but will not do so when transplanted to other sites (26, 27). The latter experiments (27) are especially pertinent since the same pituitary was studied, first as a kidney transplant, and then as one beneath the median eminence. Other evidence of a more indirect nature has been well summarized by Harris (28).

Evidence for a CNS influence that inhibits LTH secretion. Certain hypothalamic lesions in rats lead to a type of prolonged LTH secretion (29) similar to that shown by the autotransplanted pituitary (2, 3). Yokoyama and Ota (30) produced lesions in the brains of lactating rats that were associated with the ability to secrete LTH in the absence of suckling; FTH secretion could be suppressed, however, only when the rats were suckled. Their unsuckled animal was thus an experimental homologue of our intact lactator with a small litter.

Evidence that suckling suppresses FTH secretion. This has been considered in the interpretations of Experiments V, VI, VII, and VIII. That suckling effects this suppression via the CNS is a reasonable, but unproven, postulate. That the area involved is less sensitive to the effects of suckling than the one that inhibits LTH, is suggested by the fact that lactating rats with small litters may show very little evidence of FTH suppression, if any, while secreting adequate amounts of LTH.

Evidence that suckling suppresses a CNS influence that inhibits LTH secretion. Suckling must undoubtedly promote LTH secretion via a nervous pathway (21). The suppression by suckling of a CNS influence that chronically inhibits LTH secretion is an unproven postulate. It is a logical way, however, to reconcile the secretion of LTH in response to suckling, with 1) the ability of the autotransplanted pituitary to secrete LTH (2, 3); 2) the failure to explain LTH secretion by any of the other mechanisms postulated in the Introduction; and 3) the ability of certain CNS depressant drugs to initiate and maintain LTH secretion (31). The maintenance of lactation by the autotransplanted pituitary (Experiment IX) further supports this thesis.

A general explanation for the regulation of LTH secretion and its relation to follicular quiescence in the rat; the ephemerality of the corpus luteum as a reflection of this relationship. If LTH secretion is promoted by suckling in the way we have described, it should also be promoted, during other conditions, by any agent that prevents the CNS-inhibiting influence over LTH secretion from affecting the pituitary. In order to help us understand, then, the exact manner in which LTH secretion is effected during other conditions, and how in these states, also, the secretion of LTH may be associated with follicular quiescence, the explanation given previously for the effects of suckling must be modified into the following form. *The pituitary secretes LTH under only those conditions that suppress or eliminate the effect upon it of*

a CNS influence that chronically inhibits its intrinsic ability to secrete LTH. Many, but not all, of these conditions also suppress or eliminate the effect upon the pituitary of another CNS influence that specifically stimulates it to form or release FTH, and are therefore associated with quiescence of the follicular portions of the ovary. All the elements of this interpretation can be seen with diagrammatic clarity in the behavior of the transplanted pituitary, and with almost equal clarity in the activity of the lactating rat's pituitary. The following discussion will show how useful this postulation may be in analyzing the relation between LTH secretion and follicular quiescence in pseudopregnancy.

The induction of pseudopregnancy by cervical stimulation may be the result of the suppression of the activity of the CNS inhibitor over LTH secretion. A recent study demonstrated that daily stimulation of the cervix induced lactation in estrogen-pretreated rats and maintained lactation in post-parturient rats deprived of their litters (32). The effect of cervical stimulation seems to be localized to the influence that inhibits LTH, for daily cervical stimulation of castrated rats bearing ovarian autotransplants does not delay the interval to first postoperative estrus, and thus probably does not suppress FTH secretion (15). In this respect, cervical stimulation resembles minimal suckling (Fig. 7). Its effect on LTH secretion differs somewhat from sucklings', however, for cervical stimulation administered once usually induces pseudopregnancy in over 90% of rats, while if suckling is discontinued after the first or second postpartum day, slightly less than 50% of rats show a pseudopregnancy (Fig. 6).

Experiments in our laboratory have show, however, that LTH secretion in *pseudopregnant* rats continues for only a few days, when the animals are castrated (15, 33) (see also Experiment V, last paragraph). A luteal factor, therefore, probably maintains LTH secretion for the duration of pseudopregnancy. The most obvious one is progesterone. It is not only the principal ovarian hormone secreted during pseudopregnancy, but it is also responsible for the follicular quiescence of pseudopregnancy (see further below). Its ability to maintain LTH secretion would thus be consistent with our interpretation of the association between LTH secretion and follicular quiescence. (From the peculiar nature of the rat's inability to develop functional corpora lutea spontaneously, however, it must be considered capable only of maintaining LTH secretion that has already been set in progress by other factors, such as cervical stimulation, or suckling.)[5] The most suggestive findings that favor this viewpoint are these. Progesterone does not

[5] (Added in proof): I was unaware, at the time of writing, of two papers by Alloiteau and Vignal (Compt. rend. Acad. d.sci.—246: 2804. 1958; and 247: 2465. 1958) in which the maintenance of LTH secretion by progesterone was postulated. This was based on the induction of pseudopregnancy in cycling rats by progesterone or LTH given during estrus. In my own hands, however, such treatment, even with almost lethal doses of intravenous progesterone, was ineffective.

inhibit LTH secretion (33). Milk secretion is maintained for about 9 days in intact post-parturient rats deprived of their litters on day 4–5 postpartum and treated with oxytocin (34, 35, 15). If the rats are castrated at the time of litter removal, however, milk secretion soon ceases, even though oxytocin is administered every 4 hours around the clock (15); if progesterone is also administered to such castrates, however, milk secretion is maintained (15). Amounts of progesterone too small to prevent FTH release will maintain pseudopregnancy for about 20 to 25 days (15). Progesterone will induce broodiness in birds (36); this condition is associated with LTH secretion (37). It will also increase the pituitary content of LTH in rats (38); estrogen will also do this, but is more effective in the presence of the ovaries (39). Estrogen will also induce pseudopregnancy (40, 41), and will maintain corpus luteum growth (40, 42) as well as function (40) in rats. This effect of estrogen may reflect its suppression of the CNS influence that inhibits LTH secretion as well as the one that stimulates FTH; however, the evidence cited (39), plus the practical absence of estrogen during the first two-thirds of pseudopregnancy, would tend to rule estrogen out as the ovarian factor responsible for LTH secretion.

Progesterone could maintain LTH secretion by virtue of its ability to suppress the CNS inhibitor over LTH. The positive feedback aspect of this relationship implies that LTH secretion will not be maintained indefinitely, for any decrease in progesterone secretion must increase the activity of the CNS inhibitor, and thus diminish LTH secretion. This must diminish the secretion of progesterone still further, and lead eventually to the total cessation of both LTH and progesterone secretion, and to the regression of the corpus luteum. The ephemerality of the corpus luteum can thus be explained, in part, by the relationship. The specific manner in which progesterone causes follicular quiescence during pseudopregnancy fits together with this postulated progesterone-LTH relationship to explain the *variations* in the duration of luteal viability under several conditions. Let us consider these.

Progesterone prevents only the *release*, but not the formation, of FTH by the pituitary (the evidence for this is summarized elsewhere (4)). The pituitary's FTH potency, and therefore its *potential ability* to secrete FTH, thus increases under the influence of progesterone. Suckling, however, decreases the pituitary's potential ability to secrete FTH since it suppresses the *formation* of FTH. These opposite effects of progesterone and suckling on the pituitary's FTH secreting potential show a striking relationship to the duration of luteal viability. Figure 6 reflects this relationship, for the vaginal cornification that terminated each mother's interval to first estrus could not have occurred in the presence of a continued secretion of progesterone. LTH secretion, however, was maintained in all these rats except the controls, by suckling; the variations in the length of the diestrus, therefore, imply that *luteal viability, in the presence of continued LTH secretion, is*

increased in proportion to the decrease in the pituitary's potential ability to secrete FTH. This in turn suggests the possibility that FTH blocks the corpus luteum's response to LTH. The hastening of luteal regression in hypophysectomized rats treated with FTH (43, 44), the estrus-like vaginal smears of our pseudopregnant rats treated with SPG (Experiment III), the inability of suckling to prevent the occurrence of estrus in rats subjected to a strong FTH stimulus (45), the cyclic recurrence of estrus and ovulation in intact mice (5) and rats (6) bearing homotransplanted pituitaries, and the failure of corpus luteum survival in rats hypophysectomized and pituitary autotransplanted during proestrus (46) are some, at least, of the available evidences that this effect exists.

This effect would complement the operation of the positive feedback relationship between progesterone and LTH as the cause of luteal ephemerality. A diminution of progesterone secretion, for whatever reason, or progesterone's inability to prevent FTH release indefinitely (4, 15), would allow the release of FTH, which, by blocking the corpus luteum's response to LTH, would decrease progesterone still further and thus further increase FTH release. This process will progressively diminish LTH secretion when the latter is maintained only by progesterone, and thus lead to a rapid regression of the corpus luteum; if LTH secretion was maintained by suckling, however, the FTH effect could still operate, but luteal regression would proceed more slowly (compare, in Figure 6, the pseudopregnancy-like diestrous periods in the controls with the intervals to first estrus in the mothers suckling a single pup).

The respective abilities of progesterone, suckling, and pituitary transplantation to promote LTH secretion; the different effects of these factors on FTH formation and release; and the blocking effect of FTH on the corpus luteum's response to LTH, permit us to deduce the effect of their interaction on luteal viability to a remarkable degree. Thus: 1) luteal viability should be least, if LTH is practically absent, and FTH potential is high; this is undoubtedly the condition in the cycler. 2) Luteal viability should be appreciably longer, but definitely limited, if LTH is maintained by progesterone, and FTH potential is high; this is the condition in the pseudopregnant rat. 3) Luteal viability should be slightly longer, but still definitely limited, when LTH is maintained by suckling, but FTH potential is still high; this is the condition in the lactator with a small litter. 4) Luteal viability should be considerably longer, if LTH is maintained by suckling, and FTH potential is low; this is the condition in the lactator with a large litter. 5) Luteal viability should be indefinitely prolonged, if LTH is maintained by pituitary transplantation, for FTH potential will be reduced almost to zero. These relationships are set forth in Figure 17, where instead of "luteal viability" the actual observed lengths of the diestrus under these conditions are shown.

Whether LTH secretion, and with it progesterone secretion, also in-

creases in proportion to litter size is completely unknown. As litter size increased, the differences between the mean intervals to first estrus in the intact mothers and in the castrates bearing ovarian autografts tended to remain constant, or may even have decreased (Figs. 6 and 7; Table 3). This

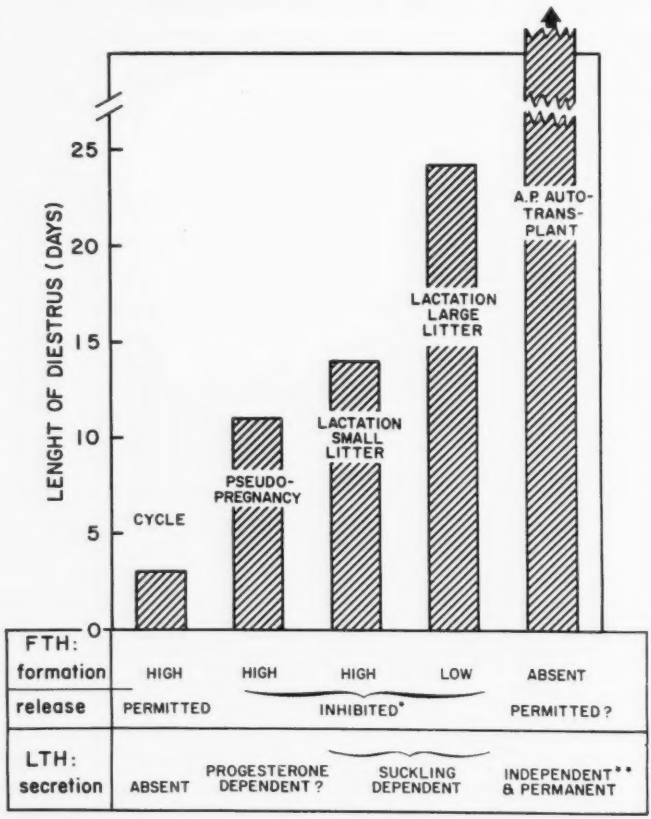

FIG. 17. The relationship between the pituitary's potential ability to secrete FTH and its secretion of LTH, on the one hand, and the duration of diestrus, as an expression of viability of the corpus luteum, on the other. Corpora lutea capable of responding to LTH are present in all conditions. The absence of LTH in the cycler is not meant to be absolute; there is probably too little LTH secreted to maintain the corpora lutea. Since practically no FTH is formed by the autotransplanted pituitary, no information is available on whether release of FTH can occur from the transplanted pituitary. The dependency of LTH on progesterone during pseudopregnancy is the author's postulate, for which suggestive evidence is outlined in the General Discussion. *FTH release is probably inhibited during lactation, as it is during pseudopregnancy; the difference between the two conditions is that FTH *formation* is also suppressed during lactation in proportion to litter size, but is not suppressed at all during pseudopregnancy (4). **LTH secretion from the transplanted pituitary is meant to be independent of CNS inhibition, and therefore "permanent." The length of the diestrus is also "permanent."

certainly suggests that progesterone secretion remained constant as the litter size increased, for in cyclers the diestrus can be lengthened in proportion to the amount of progesterone administered (15). Parkes and Bellerby showed that the amount of estrogen required for vaginal cornification in lactating mice increased in proportion to litter size (10); this phenomenon may reflect a proportionate increase in LTH secretion, but it is also explainable on the basis of the mother's increase in FTH potential with decrease in litter size, and the ability of estrogen to induce FTH release rapidly (47). Mühlbock and Boot observed an increase in the length of the diestrus induced in mice by successive implantations of large numbers of mouse pituitaries (5). This too may be the result of a progressive increase in LTH secretion, but it may also reflect the effects of repeated trauma, and the induction of mammary cancer (5) in decreasing the pituitary's ability to secrete FTH. A most urgent need is for an accurate method for directly measuring the secretion of LTH.

A detailed application of this interpretation to other species, and to other aspects of the pituitary-ovary relationship, will not be attempted here. It does seem worthwhile, however, to describe briefly a few examples of how readily such an application may be made. The length of the luteal phase of the cycle in most polyestrous mammals that have been studied is remarkably close to 14 days (48); the dog, however, which ovulates only once or twice a year, has a luteal phase of 2 months (48). These differences could be related to the maintenance of FTH potential between ovulations in the polyestrous mammals, and the opposite, in forms like the dog. The human female shows the same general relationship between the amount of daily breast feeding and the length of the postpartum amenorrhea (49, 50) that has been demonstrated in the rat between litter size and the interval to first estrus (7) (Fig. 6). This suggests that the same basic processes operate in both species. Furthermore, lactation may follow pituitary stalk section in women (51). The occurrence of galactorrhea with an amenorrhea characterized by the signs of low or absent secretion of both estrogens and gonadotrophins (52) suggests the suppression of the two CNS influences over FTH and LTH secretion; in some such cases, the presence of certain brain lesions (52) may indicate the nature of the suppression. The co-existence, in some women, of galactorrhea and ovulatory cycles resembles the condition in Yokoyama and Ota's lesioned rats (30), and supports the idea that the CNS influence that inhibits LTH secretion may be affected without involving the one that stimulates FTH secretion. This divorce between the suppressing effects of a given stimulus on both influences would explain how the dairy cow continues to ovulate during lactation (48); when one considers the wide spread in the lactating rats' intervals to first estrus in each litter size group (Fig. 6) it is not hard to imagine how such a divorce could have been accomplished by selective breeding.

A paper in preparation will present the detailed evidence for the postula-

tions outlined in the latter half of this discussion; it will also attempt to consider, to a greater degree than was possible here, the extent to which this explanation of the corpus luteum-pituitary relationship in the rat applies to other forms, and to other situations in which LTH may be secreted. The author is aware of the possible discrepancies between some of these ideas and others' findings (53, 54); these will be considered in the forthcoming paper.

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THE CORPUS LUTEUM-PITUITARY RELATIONSHIP: A STUDY OF THE COMPENSATORY HYPERTROPHY OF THE OVARY DURING PSEUDOPREGNANCY AND LACTATION IN THE RAT[1]

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ABSTRACT

The growth of an ovary remaining after unilateral ovariectomy was studied in cycling, pseudopregnant and lactating rats. The left ovary was removed in all cases on day 1 of diestrus in the cycling and pseudopregnant rats, and on day 1 postpartum in the lactating rats; the two former groups were observed up to 14 days postoperatively, the latter until day 20 postpartum. The cyclers' remaining ovaries increased in weight from operation onward. In the pseudopregnant rats there was no significant change in ovarian weight until after the 8th postoperative day, and in the lactators, for the entire observation period. These results were discussed in relation to the suppression of folliculotrophin formation or release during pseudopregnancy and lactation.

THIS study was designed to answer, at least partially, the question of what changes occur in folliculotrophin (FTH)[3] secretion during pseudopregnancy. The possible relation of these changes to the maintenance of luteotrophin (LTH)[3] secretion has been discussed at length in another paper (1). Evidence was presented in that paper that supported the thought that LTH is secreted in response to the suppression of a CNS inhibiting influence over LTH secretion (2, 3, 4); suckling probably suppresses this CNS inhibition, and is associated with follicular quiescence because suckling also suppresses a CNS stimulating influence over FTH (1). Since the specific cause of follicular quiescence might be similarly related to the cause of LTH secretion during pseudopregnancy, ovarian compensatory hypertrophy was studied, in pseudopregnant and lactating rats, as a step in determining the nature of the pseudopregnant follicular quiescence. The results obtained from these experiments, and from others still in progress, suggested that the follicular quiescence of pseudopregnancy resulted from inhibition of release, but not of formation, of FTH. The reasons for this conclusion are considered in detail in the Discussion.

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[3] See Introduction to (1) for definitions and justifications of these terms.

METHODS AND MATERIALS

The rats were 200 to 300 gram females of the Sprague-Dawley strain (Holtzman Rat Co., Madison, Wisc.). Three groups were used: 1) "cyclers"—these were selected from a large colony after having shown at least 4 regular (4–5 day) estrous cycles; 2) "pseudopregnants"—these were selected exactly as were the cyclers, but were subjected to cervical stimulation with a glass rod, on the 2 successive days of a proestrus and estrus; and 3) "lactators"—these were selected from our breeding colony only in relation to their time of delivery. The maintenance of the lactators has been described in detail elsewhere (1). The left ovary was removed from 37 cyclers and from 46 pseudopregnants on the first day of the diestrus, and from 40 lactators on the day after delivery (day 1 postpartum). Control data on the variation in weight between the right and left ovaries of intact rats were available from 102 additional rats (32 cyclers, 31 pseudopregnants, and 39 lactators). Nineteen of these lactators and 14 of these pseudopregnants were also studied for the changes in total ovarian weight during the course of lactation and pseudopregnancy.

The remaining right ovary was removed at autopsy or operation from the unilaterally ovariectomized cyclers and pseudopregnants at intervals of 1 or 2 days, up to 14 days postoperatively, and from the lactators up to 20 days postoperatively. All ovaries were weighed immediately after removal and dissection on a Roller-Smith torsion balance.

Daily, except Sunday, vaginal smears were made on all rats until removal of the second ovary. The numbers of experimental cyclers and pseudopregnants mentioned above actually represent about 90% of slightly larger numbers treated similarly. Cyclers whose postoperative vaginal cytology showed a diestrus of more than 4 days, or a pronounced departure from the preoperative pattern, were discarded. Discarded pseudopregnants were those that showed a diestrus of less than 9 days. The data include an unavoidable, but probably small, amount of error for the observation period up to roughly the 4th postoperative day, inasmuch as this period of time did not allow the diagnosis of "cycler" and "pseudopregnant" to be established beyond question. A certain number of the pseudopregnants autopsied at the longer postoperative intervals (12–14 days) had already returned to estrus. They are so noted in the Results.

Litter size in the lactating group varied from 3 to 12 pups, but in over 90% of them was 6 pups or more.

RESULTS

Cyclers and pseudopregnants. The changes in weight of the remaining ovary were evaluated in two ways: 1) its absolute weight was plotted against time after operation, its "initial" weight being taken as the mean of the 83 ovaries removed at operation (Fig. 1); 2) the weight of the remaining ovary was expressed as a percentage of the same rat's left ovarian weight; these percentages were similarly plotted against time after operation (Fig. 2). The means for a particular group of ovaries were obtained by grouping the postoperative intervals into 2-day units.

Growth of the cyclers' remaining ovaries proceeded in apparently straight line fashion throughout the experimental period. The pseudopregnants' remaining ovaries however, did not show appreciable growth until after the 8th postoperative day (day 9 of the diestrus); from this time onward, their growth was so rapid that by the 14th postoperative day they weighed almost as much as the cyclers' ovaries. Most of the rats showing

this increase in ovarian weight had returned to estrus at or just before removal of the remaining ovary (Figs. 1 and 2).

The mean weight of a single ovary on the 7th diestrous day of 14 intact pseudopregnant rats was 29.0 mg. The mean left ovarian weight of the 83 pseudopregnants and cyclers on the 1st diestrous day was 35.4 mg. (Fig. 1). This difference (18% decrease) is somewhat less than that observed in the lactating rat during approximately the same period of time postpartum (Fig. 3), but it suggests that an ovarian weight decrease of this order of magnitude occurs normally in pseudopregnancy. If this is so, then the remaining ovary's *maintenance* of its "initial" weight through the first 9 days of pseudopregnancy (Figs. 1 and 2) may reflect a very small amount of growth.

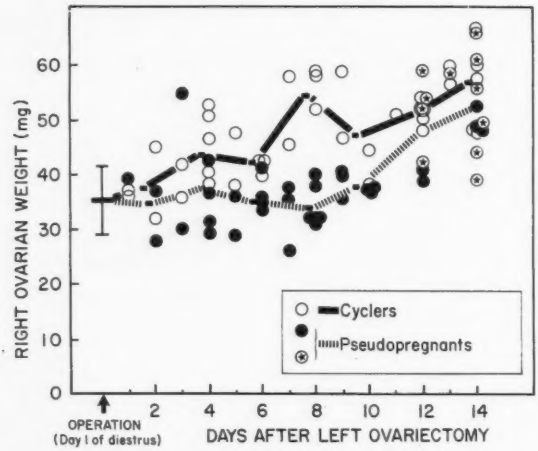

FIG. 1. The change in weight of the right ovary of cyclers and of pseudopregnants, in the period of 14 days following removal of the left ovary. The horizontal bar and vertical line at "operation; day 1 of diestrus" represent the mean and standard deviation of the 83 left ovaries removed at operation, and can be taken as the theoretical original weight of the remaining right ovary. Actually, in 102 rats of various types (see Methods) the mean right ovarian weight was 4.4% higher than the mean left ovarian weight; even if the theoretical weight of the remaining right ovary is corrected for this variation, however, the results would not be essentially different. The values for the pseudopregnants represented by a star within a circle are from those rats that had returned to estrus at, or very shortly before, removal of the remaining ovary.

Lactators. The rats' ovaries decrease in weight during lactation (5). The progressive change in weight of the remaining ovary of the hemiovariectomized lactators, therefore, was compared directly with the changes in ovarian weight of intact lactators (Fig. 3). No significant difference could be observed between the two curves.

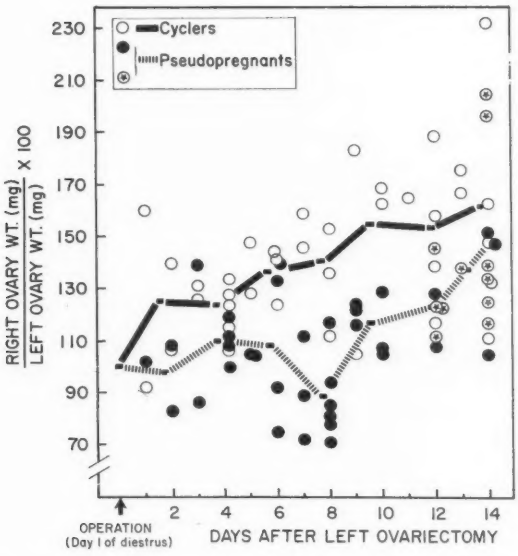

FIG. 2. Exactly the same symbols and data shown in Figure 1, but each remaining right ovarian weight is expressed as the percentage of the same animal's left ovarian weight.

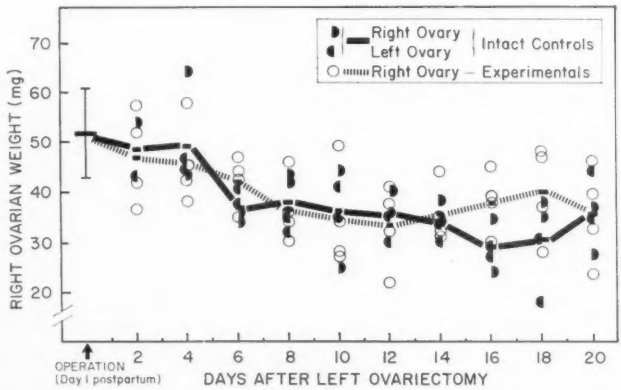

FIG. 3. The change in weight of the ovaries of intact, and of left-ovariectomized lactating rats, during the course of lactation. The horizontal bar and vertical line at "operation; day 1 postpartum" represent the mean and standard deviation of the 40 left ovaries removed at operation. The "experimentals" are, of course, the left-ovariectomized rats.

DISCUSSION

The ovaries of pseudopregnant, and of lactating rats show the same growth response to exogenous FTH as do those of cyclers (1). We can therefore eliminate, as the cause of follicular quiescence in either pseudopregnancy or lactation, the effects of either LTH itself or the conditions of its secretion, on the ovary's response to FTH. The failure of ovarian compensatory hypertrophy must therefore be due to the absence or depression of FTH stimulation to the ovary.

In lactation this is apparently due to the suppression of FTH secretion by suckling (1, 6, 7). In pseudopregnancy, however, the pituitary, when assayed by the mouse uterine weight method, shows a progressive increase in FTH potency until the 7th diestrous day; this potency remains then at a level roughly twice that of the cycler's pituitary until the first proestrus, when it falls back to the original level (8). These findings are similar to those reported for the content of "ovulating hormone" in the rabbit's pituitary during pseudopregnancy (9), and also agree with the ability of the pseudopregnant rat to ovulate in response to electrical stimulation of the brain (10). The absence of compensatory hypertrophy of the ovary during the first 9 days of pseudopregnancy, therefore, suggests that *only* the release of FTH is prevented. This interpretation is also supported by the following observations. When the pseudopregnant rat is castrated on the first diestrous day and portions of its ovary transplanted to the kidney (this involves loss of all viable luteal tissue (11)), it shows a 5 to 6 day interval to first estrus postoperatively; the cycling rat subjected to the same procedure shows exactly the same interval (12). The lactating rat suckling a 2-pup litter subjected to this procedure returns to estrus in 7 days, yet the *intact* lactator with a 2-pup litter has an average interval to first estrus of 16 days (1). The suckling of a 2-pup litter does not appreciably suppress FTH formation, although it is a sufficient stimulus for the maintenance of LTH secretion (1).

The factor responsible for the inhibition of FTH release is undoubtedly progesterone; its ability to delay ovulation is well known, and in sufficient amounts (5.0 mg./day), its administration to intact cyclers results in an increase in pituitary FTH potency roughly equivalent to that seen during pseudopregnancy (12). Even in high doses (10.0 mg./day), it does not depress pituitary FTH potency in castrated rats (12). The degree of inhibition of FTH release is probably far from absolute, however. The possible *maintenance* of ovarian weight in the hemiovariectomized pseudopregnants, compared to the slight loss in weight observed in intact pseudopregnants, suggests this (see Results, and Figs. 1 and 2). In another study (13), we have found that rats suckling 2-pup litters show only about half the ovarian weight loss shown by lactators with large litters; that this reflects some degree of inhibition of FTH release, however, is indicated from the fact that

post-parturient rats, deprived of their litters shortly after delivery, maintain the ovarian weight reached by the end of pregnancy (13). Moreover, the inhibition can apparently be overcome by a stimulus of sufficient magnitude, since electrical stimulation of the brain of pseudopregnant rats will induce ovulation (10). The only available data on the duration of progesterone's effect on FTH release comes from work in progress in this laboratory. We have found, in pseudopregnant rats treated with 10.0 mg./day of progesterone, that vaginal mucification first appears after 14 days of treatment; it is absent at 21 days, and again present after 28 days of treatment. These findings are being pursued further, but they strongly suggest a periodic release of at least enough FTH to stimulate estrogen secretion, and, therefore, that there is a definite limit to the duration of this inhibiting effect of progesterone. The rapid increase in ovarian weight from day 9 onward, in the unilaterally ovariectomized pseudopregnants (Figs. 1 and 2), probably reflects this diminishing inhibition of FTH release.

As a result of the inhibition of FTH release by progesterone, the pituitary's *potential ability to secrete* FTH during pseudopregnancy must be increased. The implications of this, together with the fact that progesterone does not inhibit the secretion of LTH (14), for the way in which LTH secretion is promoted during pseudopregnancy, and for the causes of luteal ephemerality in general, have been considered in another paper (1).

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THE CORPUS LUTEUM-PITUITARY RELATIONSHIP: THE LUTEOTROPHIC ACTIVITY OF HOMOTRANSPLANTED PITUITARIES IN INTACT RATS[1]

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ABSTRACT

The effect of a pituitary homotransplanted beneath the kidney capsule on the estrous cycle of the intact rat was studied. The majority of 37 rats treated in this way showed a pseudopregnancy-like prolongation of the diestrus in progress at the time of operation, with a marked tendency for succeeding cycles, over a period of roughly 3 months, also to be prolonged. Eight control rats, homotransplanted with a piece of cerebellum in place of a pituitary, tended to continue to have estrous cycles of normal length. The prolonged cycles had an immediate onset in rats operated on at any stage of the vaginal cycle except the second diestrous day. In these, cycle prolongations began after the first complete postoperative cycle. There was a high incidence of incomplete estrous smears, characterized by only a single day of nucleated or cornified epithelial cells and preceded and followed by smears predominantly of leucocytes, in the experimental group. The incidence of such smears ending normal as well as prolonged cycles was greater than in the control rats. These results were briefly discussed in relation to the pituitary factors that may affect the life of the corpus luteum, and the control of LTH secretion.

INTRODUCTION

IN THE course of a study of the effects of oxytocin on the secretion of luteotrophin (LTH)[2] in the rat (1), a control experiment, involving the effect on the estrous cycle of a single rat pituitary homotransplant, was carried out. The rats treated in this way showed a prolongation of the cycle in progress to the length of a pseudopregnancy; controls implanted with cerebellar tissue continued to cycle normally. This finding seemed worthy of more study, and we accordingly set up a large colony of such rats, with suitable controls, to determine a) the reproducibility of this phenomenon; b) the relation between the stage of the host's estrous cycle at operation and the time of onset of the cycle prolongation; and c) the repetitiveness of such prolonged cycles. Similar experiments in mice (2, 3) came to our atten-

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[2] See Introduction to (4) for the definition of this term.

tion after most of this program had been carried out. Our own study supplements these by extending the observations to the rat; both studies offer valuable information regarding some of the factors that affect the viability of the corpus luteum, as well as the question of whether LTH secretion is a continuous process, or one induced by specific stimuli. These are discussed briefly in this paper, and at greater length in another paper (4).

METHODS

Eighty-two Sprague-Dawley female rats of 180 to 300 gms. body weight (Holtzman Rat Company, Madison, Wisc.) were selected after each had shown, by vaginal smears, at least 4 normal (4 to 5 day) estrous cycles. Thirty-seven served as pituitary donors, an equal number as pituitary hosts, and 8 as control hosts. The donors were killed with ether on day 1 of a diestrus. The hosts were operated on during proestrus, estrus or the first or second day of diestrus. At operation the left kidney was exposed through a flank incision and a small pocket made under the kidney capsule. The donor's pituitary was removed immediately after death, and, with only a few minutes' delay, placed within the host's kidney capsule pocket. The 8 control hosts were operated on similarly, but received, in place of a pituitary, a piece of cerebellum (approximately the same size as the pituitary) from each of 8 of the donor rats (for further details concerning transplantation, see (4)).

All animals were followed by daily, except Sunday, vaginal smears for a minimum of 28 and a maximum of 121 days. At the completion of the experiment, all the controls, and 34 of the 37 experimental rats were killed with ether, and the ovaries, uteri and *in situ* pituitary removed, weighed, and fixed in Bouin's solution. The kidney containing the transplanted pituitary was fixed in toto in Bouin's also. Both pituitaries were later sectioned at 10 μ; alternate slides were stained with Harris' hematoxylin and eosin, and with Azan triple stain. In 4 animals, the transplanted pituitaries could not be recognized as such at autopsy; these were diagnosed as "non-viable," since a careful search of the surrounding tissues failed to uncover the pituitary in an ectopic site. The remaining transplants were readily recognized; the overlying capsule had a rich supply of small blood vessels running to the gland, which was raised above the surface of the kidney. These were diagnosed as "viable." The microscopic appearance of these glands is described in the Results.

The rat's vaginal cycle (5) is so well known that it need not be described here in detail. For the purpose of analyzing our results, day 1 of the cycle was considered as the day of the proestrus (nucleated epithelial cells), or estrus (cornified epithelial cells), if the latter followed a diestrous day (leucocytes). Cycle length was the total number of days, inclusive, from day 1 to the last diestrous day before the beginning of the next cycle. In many of the cycles, particularly in the experimental group, day 1 consisted of a smear of nucleated or cornified cells, and day 2 of leucocytes; these will be designated in the results as "incomplete estrus" smears, to distinguish them from the normal sequence of nucleated, cornified and leucocyte smears on days 1, 2, and 3, respectively.

RESULTS

Four hundred and sixty-three cycles, varying in length from 2 to 120 days, were observed in the 37 pituitary-hosts (experimentals) and the 8 control rats. We have classified these as follows: *Normal or unaffected:* 3 to 7 days duration; *intermediate:* 8 to 10 days duration; *pseudopregnancy-like:* 11 to 21 days duration; *prolonged:* longer than 21 days. Though some of

TABLE 1. THE EFFECT ON THE ESTROUS CYCLES OF INTACT RATS OF EITHER PITUITARY OR CEREBELLUM HOMOTRANSPLANTATION

Treatment	Number of rats	Duration observed (days/rat)	Total number of cycles observed	Type and incidence of cycle[1] following treatment							
				Normal		Intermediate		Pseudopregnancy-like		Prolonged	
				No.	%	No.	%	No.	%	No.	%
Pituitary homotransplantation	37	28–121	333	147	44.1	34	10.2	137	41.1	15	4.5
Cerebellum homotransplantation	8	80–100	130	113	86.9	10	7.7	7	5.4	0	0.0

[1] See *Results*, first paragraph, for a definition of the types of cycles observed following treatment.

this classification is obviously arbitrary, it helps to define the main effects of the experimental procedure.

The distribution of these cycles in the experimental and control groups is shown in Table 1. Of 333 experimental cycles, 41% were pseudopregnancy-like, and 44% were unaffected; 87% of the control cycles were unaffected and only 6% were pseudopregnancy-like. The effect of pituitary transplantation became somewhat clearer when the comparison was made on the basis of all cycles longer than 7 days, as well as on the basis of the incidence of pseudopregnancy-like cycles only, and with the rats bearing viable pituitary grafts separated from rats with non-viable ones. In the latter, and the controls, pseudopregnancy-like cycles were 10% and 6% of tht total, respectively, while in the former group, their incidence was 53%. In the animals bearing viable grafts, 69% of the cycles were longer than 7 days; this incidence was 28%, in those bearing a nonviable graft, and 13% in the controls (Table 2).

The relation between the onset of cycle prolongation and the estrous cycle stage at which the homotransplantation was done is shown in Table 3. When the host was operated on during either proestrus, estrus or day 1 of diestrus, the cycle then in progress was, in most cases, lengthened; when the operation was done on day 2 of diestrus, the cycle in progress tended, in most cases, to be of normal length, but was followed by a lengthened cycle.

The incidence of "incomplete estrus" smears (see Methods), and their relation to the type of preceding cycle is shown in Table 4. The experi-

TABLE 2. THE INCIDENCE OF CYCLES LONGER THAN 7 DAYS IN RATS WITH VIABLE AND NON VIABLE PITUITARY HOMOTRANSPLANTS, AND RATS WITH CEREBELLUM HOMOTRANSPLANTS

Group	Number of rats	Total number of cycles	Cycles following homotransplantation			
			Normal[2]		Longer than 7 days[3]	
			No.	%	No.	%
Rats with autopsy evidence of viable pituitary transplant	30[1]	236	71	30.1	165	69.9
Rats with autopsy evidence of non-viable pituitary transplant	4[1]	61	44	72.1	17	27.9
Rats with cerebellum homotransplants (controls)	8	130	113	86.9	17	13.1

[1] Three of the 37 experimental rats were not autopsied.
[2] Cycles of 3 to 7 days duration. See Results, first paragraph.
[3] Intermediate, pseudopregnancy-like, and prolonged cycles. See Results, first paragraph.

TABLE 3. THE RELATION BETWEEN THE ESTROUS CYCLE STAGE AT THE TIME OF PITUITARY HOMOTRANSPLANTATION INTO INTACT RATS, AND THE TIME OF ONSET OF CYCLE PROLONGATION

Estrous cycle stage at homotransplantation	Number of rats	Effects of treatment			
		Rats with *first* postoperative cycle longer than 10 days		Rats with *second* postoperative cycle longer than 10 days	
		No.	% of total	No.	% of total
Proestrus	13	11	85	7	54
Estrus	8	7	88	4	50
Diestrus day 1	9	7	78	7	78
Diestrus day 2	7	2	29	7	100

mental group showed a higher incidence of these smears than did the controls. This was in part related to their higher incidence of pseudopregnancy-like or longer cycles, but was also, in part, a characteristic of the group as a whole, since the frequency of such smears, even after unaffected cycles, was greater than in the controls.

The microscopic appearance of the transplanted pituitaries was much as has been previously described by Everett (6, 7). The transplants were well vascularized and had a central fibrous core. The cells were predominantly eosinophils, and no basophils were seen. Although the pituitaries *in situ* showed a predominance of eosinophils, they contained a very definite, though moderate, number of basophils as well.

DISCUSSION

Nikitovitch-Winer and Everett (8) have shown that hypophysectomy and pituitary autotransplantation resulted in the typical prolongation of corpus luteum activity, in rats operated on in any stage of the estrous cycle except proestrus. In our study the rats operated on during the 2nd day of diestrus were the only ones that did not show a characteristic immediate lenthening of the cycle in progress. This difference can perhaps be explained

TABLE 4. THE DISTRIBUTION OF CYCLES ENDING IN AN INCOMPLETE ESTROUS SMEAR, ACCORDING TO THE TYPE OF PRECEDING CYCLE, IN INTACT RATS HOMOTRANSPLANTED WITH EITHER A PITUITARY OR A PIECE OF CEREBELLUM

Type of cycle[1]	Pituitary homotransplants (37 rats)			Cerebellum homotransplants (8 rats)		
	Total no. of cycles	Cycles ending in incomplete estrus		Total no. of cycles	Cycles ending in incomplete estrus	
		No.	% of total		No.	% of total
"Normal"	147	31	21.1	113	1	0.9
"Intermediate"	34	13	38.2	10	1	10.0
"Pseudopregnancy-like"	137	51	37.2	7	1	14.3
"Prolonged"	15	7	46.7	0	—	—
All cycles	333	102	30.6	130	3	2.3

[1] For definition of cycle types, see Results, first paragraph

as follows. During proestrus, the corpora lutea of the preceding ovulation lose their ability to respond to LTH. When hypophysectomy and pituitary autotransplantation is done at this time ovulation does not occur and the animal is thus left with no viable corpora. When a pituitary is homotransplanted into an intact rat during proestrus, however, ovulation occurs at the expected time, and the new crop of corpora lutea are then maintained by the graft's LTH. The corpora lutea on day 2 of diestrus, though capable of responding to LTH (8) are probably not stimulated soon enough by the homotransplanted pituitary to prevent ovulation from occurring at the expected time. The pseudopregnant cycle thus begins with the next ovulation.

A somewhat similar phenomenon has been described for the induction of pseudopregnancy in rats by cervical stimulation. Greep and Hisaw (9) stimulated the cervices of rats during either estrus, or on day 1 or day 2 of diestrus. Of those stimulated in estrus 86% showed an immediate pseudopregnancy, while the remainder cycled normally. Stimulation on day 1 of diestrus resulted in a 54% incidence of immediate pseudopregnancies, while 31% became pseudopregnant after completing the cycle in progress. When stimulation was done on day 2, however, only 16% became pseudopregnant immediately, while 56% did so after completing the cycle in progress. DeFeo (10) has noted the same phenomenon, although his incidences of immediate pseudopregnancies were greater than those observed by Greep and Hisaw (9). In our own laboratory, 8 rats were cervically stimulated on day 3 of diestrus; each showed proestrous and estrous smears on the succeeding two days, respectively, and 7 then went into a pseudopregnant cycle. These findings, in general, fit rather well with the effects of pituitary homotransplantation, and with our interpretation of them.

Specific tests of luteal activity, aside from the evidence of cycle prolongation, were not made. However, the similarity between our results and those of Mühlbock and Boot (2, 3), and the demonstration that deciduoma formation occurred during the prolonged cycle (3), make it highly likely that the homotransplanted rat pituitary produced the effects it did by maintaining the corpora lutea. These results, together with those of other experiments (4), and of Nikitovich-Winer and Everett (8), suggest strongly, therefore, that LTH secretion by the *in situ* pituitary in the rat and mouse must be a discontinuous process, since, if it was not, the capacity of the corpora to respond to LTH at all stages of the cycle except proestrus, should allow functioning corpora to develop at each ovulation.

The cyclic recurrence of complete or incomplete estrous smears, even though LTH secretion from the pituitary transplant was undoubtedly continuous, probably reflected the influence of the folliculotrophins (FTH)[3]

[3] A generic term for FSH and LH. See Introduction to (4) for a justification for this term.

upon the corpora and the follicles. Mühlbock and Boot demonstrated that ovulation occurred in their mice during these periods (3). Progesterone secretion must have diminished at these times, for estrogen will not cornify the vagina in the presence of sufficient progesterone. The corpora of the hypophysectomized rat bearing an autotransplanted pituitary apparently secrete such amounts of progesterone, since even large doses of estrogen will not cornify the vaginas in such rats (7). In the intact rat bearing a homotransplanted pituitary the inability of the corpora to maintain a constant level of progesterone secretion must therefore have reflected a blocking effect of FTH on their response to LTH. The frequent "incomplete estrus" smears, however, suggest that, in the presence of a continuous source of LTH, this blocking effect may have been masked by the secretion of progesterone by the new crop of corpora lutea. Studies in progress, in hysterectomized rats bearing homotransplanted pituitaries, have in fact shown that at any stage of the prolonged diestrus that these rats exhibit, only one set of large, pink corpora lutea are visible in the ovaries (11). Thus, even if ovulation was not reflected in a complete estrous smear, it must have been associated with the eventual regression of the old corpora. Other evidence for the blocking effect of FTH on the corpora, and its significance as one of the factors that limits their viability, have been considered in more detail in another paper (4).

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THE CORPUS LUTEUM-PITUITARY RELATIONSHIP: THE LACK OF AN INHIBITING EFFECT OF PROGESTERONE ON THE SECRETION OF PITUITARY LUTEOTROPHIN[1]

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ABSTRACT

Neither hemiovariectomy nor the administration of 2.0 or 4.0 mg./day of progesterone for 8 days affected the size of the corpora lutea of pseudo-pregnant rats. The same lack of effect of hemiovariectomy, or of treatment with progesterone (5.0 or 10.0 mg./day for 28 days) on the size of the corpora lutea was seen in hypophysectomized rats bearing autotransplanted pituitaries. These results, in comparison with those of other target organ hormones on the secretion of their pituitary trophins, were interpreted as evidence that the progesterone-LTH relationship is not one of negative feedback.

THE inhibition of secretion of a pituitary trophin by its target organ hormone is one of the important processes through which the secretion rate of the trophin is regulated. This type of control—which resembles negative feedback in physical systems—is exerted by the adrenal cortical hormones, over ACTH secretion, by thyroid hormone, over TSH secretion, and by the estrogens and androgens under certain conditions, over folliculotrophin (FTH)[2] secretion. The site of action of the target organ hormone could be directly on the pituitary, or on a CNS area that stimulates the secretion of these trophins (1). If the pituitary is separated from the CNS by transplantation, it practically ceases to secrete FTH (2, 3). This makes it impossible to test the direct effect of estrogens on the pituitary, but it also suggests that these hormones probably act primarily on a CNS area that stimulates FTH secretion. Evidence for this has been cited elsewhere (4). A small amount of ACTH may be secreted by the transplanted pituitary (5, 6) but no information is available on whether the adrenal cortical steroids inhibit this secretion. An appreciable, though definitely subnormal, amount of TSH is secreted by the transplanted pituitary (6, 7); the available evidence makes it almost certain that the thyroid hormone may directly inhibit this secretion (6, 7, 8, 9). Thus, in the case of those pituitary trophins that are subject to a "negative feedback" type of

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[2] FSH and LH; see the Introduction to (4) for the definition and justification of this term.

control by their target organ hormones, it is apparent that: 1) the major, or entire, source of stimulation for secretion of the trophin arises in the CNS; and 2) the target organ hormone may suppress the activity of this CNS area, as well as the pituitary's slight intrinsic ability to secrete the trophin.

The transplanted rat pituitary secretes luteotrophin (LTH),[3] however, at rates that are probably equal to those obtaining in the intact animal (2, 3, 4). This implies the possibility that LTH secretion is an intrinsic property of the pituitary, which is *inhibited* by a CNS influence. Everett first suggested the latter possibility, in order to explain the behavior of the transplanted pituitary (2, 3); evidence in support of this is presented in another paper (4), as well as in a recent study by Barraclough and Sawyer (10). If the progesterone-LTH relationship was one of "negative feedback," progesterone could conceivably inhibit LTH secretion, either by acting at the CNS level to increase the activity of the CNS inhibiting influence, or at the pituitary level, by reducing its intrinsic ability to secrete LTH. In either case, the administration of large amounts of progesterone either to pseudopregnant rats, or to hypophysectomized rats bearing an autotransplanted pituitary should prevent the corpora from growing, while reduction of the number of corpora in such rats, in the absence of exogenous progesterone, should increase their growth. Conversely, if the system was not one of "negative feedback," these procedures should not inhibit the growth of the corpora lutea. The studies described in this paper were undertaken to test these possibilities.

MATERIALS AND METHODS

All rats were of the Sprague-Dawley strain (Holtzman Rat Co., Madison, Wisc.). Body weights were between 250 and 300 gm. Only those rats were used that had shown by daily (except Sunday) vaginal smears a succession of at least 4 normal (4–5 day) estrous cycles. The techniques of hypophysectomy and pituitary autotransplantation, and the criteria used to judge completeness of hypophysectomy, were exactly as described elsewhere (4).

The endpoint of maintained LTH secretion was corpus luteum size, which was judged from the weight of the fixed organ. At autopsy, the ovaries, after dissection and weighing, were placed in Bouin's fluid. After at least a week's fixation the individual corpora were dissected from the ovary, under a layer of Bouin's fluid, with the aid of dissecting binoculars. They were then weighed, usually as a set from each ovary, and the results expressed as the mean corpus luteum weight per rat. Weighing was done in the pseudopregnancy experiment, with a 25 mg. scale Roller-Smith torsion balance (sensitive to 0.01 mg.), and in the other experiments with a Mettler balance, type M5 (sensitive to 0.005 mg.). The results of weighings on the two balances agreed with one another, within a possible error of less than 10%.

Progesterone[4] was injected once per day, in a volume of 0.2 cc. of sesame oil. The details of duration of administration and doses are given with the individual experiments.

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[3] See Introduction to (4) for the definition of this term.

[4] The supplies of progesterone used in this study were donated through the kindness of Dr. Edward Henderson, of the Schering Corporation.

The corpora lutea were prepared for microscopic examination, after embedding in paraffin, by sectioning them at 10 μ, and staining with Harris' hematoxylin and eosin.

RESULTS

Experiments in pseudopregnant rats. Pseudopregnancy was induced by mechanical stimulation of the cervix during proestrus and estrus. The rats were divided into 3 groups: 1) intact and uninjected; 2) hemiovariectomized and uninjected: and 3) intact rats treated with progesterone. The details of the experimental conditions, and the results obtained, are set forth in Table 1. In this experiment, no significant change in corpus luteum size followed either the reduction of the total number of corpora, or the administration of progesterone. The rate of growth of the corpus luteum during pseudopregnancy was reflected in the weights obtained at days 1, 4, and 10 in the hemiovariectomized groups.

TABLE 1. THE EFFECT OF PROGESTERONE, OR OF HEMIOVARIECTOMY, ON CORPUS LUTEUM SIZE, IN PSEUDOPREGNANT RATS

Experimental conditions[1]	Number of rats	Mean No. corpora lutea per rat	Corpus luteum weights per rat (mg.)					
			On day 1		On day 4		At autopsy	
			Mean	Range	Mean	Range	Mean	Range
I. Intacts; autopsy on day 9	6	8.8	—		—		*1.25*	1.02–1.42
II. Intacts; autopsy on day 10	6	9.0	—		—		*1.21*	1.02–1.36
III. Hemiovariectomy on day 1; autopsy on day 10	3	6.0[2] 5.0	*0.66*[2]	0.61–0.69	— —		— *1.23*	1.18–1.26
IV. Hemiovariectomy on day 4; autopsy on day 10	4	4.0[2] 4.5	— —		*1.05*[2] —	0.96–1.25	— *1.20*	1.10–1.28
V. Intacts; progesterone 2.0 mg./day from days 1–8; autopsy day 9	3	8.3	—		—		*1.23*	1.21–1.28
VI. Intacts; progesterone 4.0 mg./day from days 1–8; autopsy day 9	3	9.3	—		—		*1.32*	1.21–1.45

[1] Days referred to are, in all cases, those of the pseudopregnant diestrus.
[2] From ovary removed at operation.

Experiments in hypophysectomized, and in hypophysectomized, pituitary-autotransplanted rats. The design of this experiment was essentially the same as the previous one. Three groups of both types of hypophysectomized rats were observed: 1) ovaries intact, and uninjected; 2) hemiovariectomized, and uninjected; and 3) ovaries intact, and injected with progesterone. All animals were killed on the 28th day after operation. All operations were done on day 1 of the diestrus, in normal cycling rats. Progesterone injections were started on the day of operation and were continued daily for 28 days. The further details of this experiment, and the results are shown in Table 2.

In the hypophysectomized rats without pituitary autotransplants, the mean corpus luteum weights of the rats with intact ovaries, the hemiovariectomized rats, and the progesterone treated rats (5.0 mg./day), were similar (Table 2). The mean for the entire group was 0.80 mg., with a standard deviation of 0.31 mg. (Table 2).

The hypophysectomized rats bearing pituitary autotransplants and intact ovaries had a mean corpus luteum weight of 1.85 mg.; in the hemiovariectomized rats this value was 1.74 mg. The means for the rats treated with 5.0 or 10.0 mg. of progesterone/day were 2.02 and 2.08 mg., respectively (Table 2).

Histology of the corpora lutea. The luteal cells of the hypophysectomized animals without autotransplanted pituitaries showed typical regressive changes (Fig. 1, A). Those of the animals bearing pituitary autotransplants, *regardless of whether they received exogenous progesterone* (Fig. 1, B, C, D) were typical of the healthy, actively secreting corpora lutea described by Everett (2, 3).

Completeness of hypophysectomy. Of a total of 41 rats, the 36 listed in

TABLE 2. EFFECT OF PROGESTERONE TREATMENT OR OF HEMIOVARIECTOMY ON CORPORA LUTEA OF HYPOPHYSECTOMIZED RATS BEARING AUTOTRANSPLANTED PITUITARIES

Experimental group	No. of rats	Mean No. of corpora lutea/rat	Corpus luteum wt. (mg.)	
			Mean	Range
I. Hypophysectomy alone	4	14.5	*0.80*	0.59–1.00
II. Hypophysectomy alone, plus hemiovariectomy	3	4.7	*0.71*	0.30–1.27
III. Hypophysectomy alone, plus progesterone 5 mg./day ×28 days	5	12.4	*0.86*	0.38–1.16
All Hypophysectomy Alone Groups	12	11.2	*0.80*	0.30–1.27[1]
IV. Hypophysectomy plus pituitary autotransplantation	7	13.0	*1.85*	1.58–2.03
V. Hypophysectomy plus pituitary autotransplantation, plus hemiovariectomy	6	7.8	*1.74*	1.43–1.89
VI. Hypophysectomy plus pituitary autotransplantation, plus progesterone 5 mg./day ×28 days	5	12.6	*2.02*	1.90–2.14
VII. Hypophysectomy plus pituitary autotransplantation, plus progesterone 10 mg./day ×28 days	6	9.8	*2.08*	1.71–2.31

[1] Standard deviation =0.31 mg.

Table 2 satisfied the criteria of completeness of hypophysectomy described elsewhere (4). The possibility of incomplete hypophysectomy was noted at operation for each of the 5 rats found to have remaining pituitary remnants at autopsy, while the complete gland was accounted for at operation, for every one of those designated as "complete" (4). The adrenal weights in the 5 "incompletes" were: 30.8, 36.0, 37.4, 43.0 and 46.0 mg. The mean adrenal weight of the 12 "completely" hypophysectomized rats without autotransplanted pituitaries was 25.1 mg. (range: 21.8–28.6), and of the 24 "completely" hypophysectomized rats bearing autotransplanted pituitaries, 27.4 mg. (range: 24.0–31.4). The mean adrenal weight of untreated cyclers of equivalent body weight in our colony is 65.0 mg., and the lower limit of the range is 50.0 mg.

The gross appearance of all ovaries in the "completely" hypophysectomized rats bearing autotransplanted pituitaries was exactly as described by Everett (2, 3). Under the microscope, the ovaries of these rats, and of the "completely" hypophysectomized rats without transplants showed only primordial and small primary follicles, and atrophic interstitial tissue.

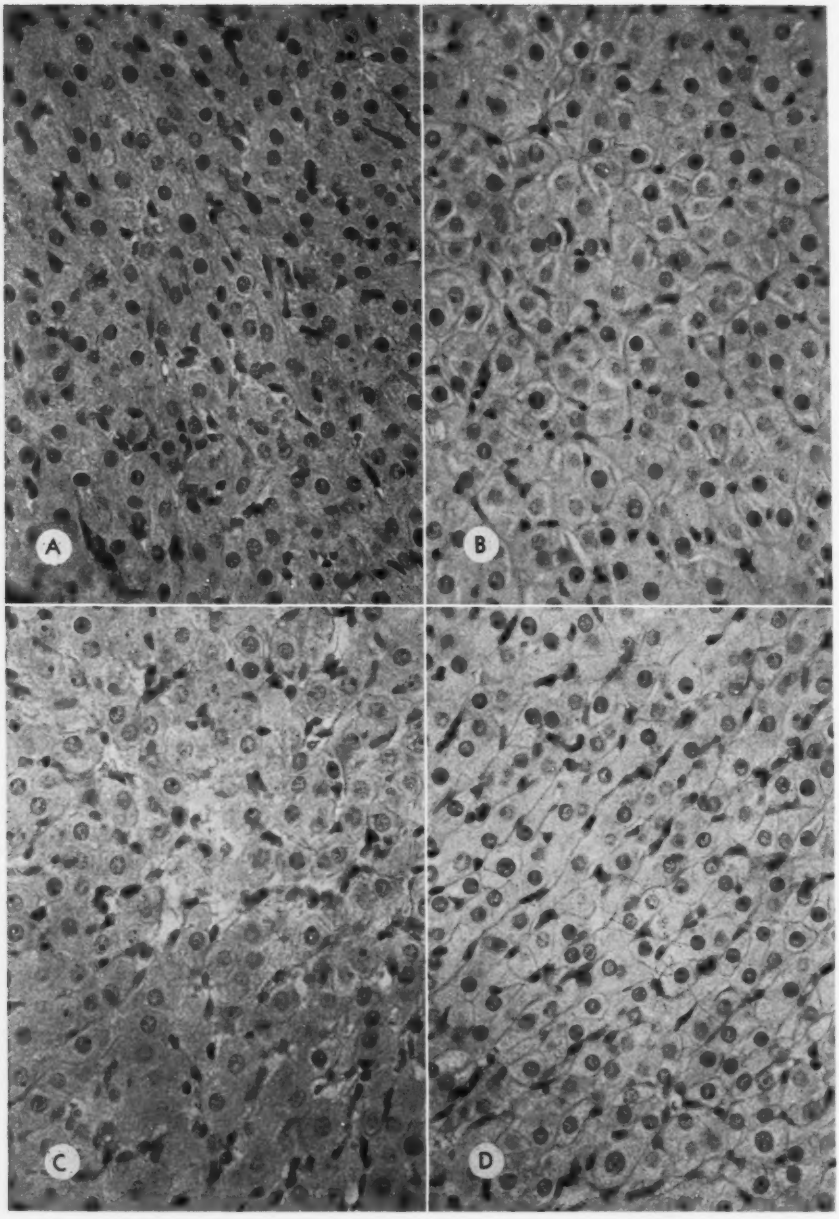

FIG. 1. High power (×333) photomicrographs of the corpora lutea of hypophysectomized rats, with or without autotransplanted pituitaries or treatment with progesterone. A) Hypophysectomy without autotransplantation or progesterone; B) Hypophysectomy plus autotransplantation, without exogenous progesterone; C) Like B, but with 5.0 mg. of progesterone/day; D) Like B, but with 10.0 mg. of progesterone/day. All animals were killed 28 days after hypophysectomy; progesterone treatment was begun on the day of operation and continued daily through this period.

DISCUSSION

The inhibiting effect of a target organ hormone on the secretion of its particular pituitary trophin ("negative feedback") is reflected in two general phenomena: 1) removal of the target organ results in signs of increased secretion of the trophin; and 2) administration of the target organ hormone results in signs of decreased secretion of the trophin. Typical of the latter is the regression of the target organ; this is rapid in onset, progressive with duration of treatment, and proportional to the dose of target organ hormone administered. *Both* of these phenomena characterize the relationships between the thyroid hormone and TSH, the adrenal cortical steroids and ACTH, and the estrogens and FTH, particularly FSH. An *apparent* exception to this generalization is the maintenance of ovarian weight, under certain circumstances, by the estrogens; this is due, however, to their ability to induce ovulation (11, 12), to maintain the corpora lutea (13, 14), and to stimulate directly a certain degree of follicular growth (15, 16), and not to a lack of inhibition of the signs of FSH secretion.

The *actual* exception to the rule of these relationships is that of luteal progesterone and pituitary LTH in the rat. As the following discussion will show, both of the characteristic features of "negative feedback" are absent from this relationship.

Let us consider first whether weight is an adequate parameter of size. The histology of the corpora (Fig. 1) permits us to see that weight expresses differences in the amount of healthy appearing luteal cells. There is also a close parallel between differences in weight (Tables 1 and 2) and differences in volume (volume being calculated from the published data on corpus luteum diameter) of the corpora lutea in the cycle and pseudopregnancy (17), and of hypophysectomized rats bearing, or lacking, a transplanted pituitary (2, 3).[5] Weight is thus undoubtedly an adequate parameter of size.

The data thus demonstrate that a) the corpora do not grow, or may slowly regress, in the absence of the pituitary (18, 19, 20) (compare III and IV, in Table 1 with I in Table 2); b) the corpora grow steadily under the influence of the transplanted pituitary (2, 3), as well as during the course of pseudopregnancy (17) (Table 1, and Table 2, IV); c) progesterone, in the absence of the pituitary, does not greatly alter (if at all) the regression of the corpora (Table 2, I and III); and d) in the presence of a transplanted pituitary, progesterone does not prevent the corpora from growing (Table 2, IV, VI and VII). These facts therefore indicate that progesterone does not induce signs of decreased secretion of LTH.

The following indicates that the progesterone-LTH relationship also

[5] The shape of the rat's corpus luteum is almost spherical, so that the volume can be calculated from the formula: volume $= \frac{1}{6}\pi d^3$. Although no measurements were made in this study of corpus luteum weight during the estrous cycle, the values given in Table 1 for day 1 and day 4 of pseudopregnancy are probably close enough to those of cycling rats for comparison with Long and Evans' values (17).

fails to show the signs of increased trophin secretion following removal of the target organ. The lack of compensatory hypertrophy of the corpora lutea (Table 1, I, II, III, IV and Table 2, IV, V) suggests the absence of this feature of "negative feedback." More conclusive evidence comes from the following three experiments, the first two of which are unpublished. 1) Castration and ovarian autotransplantation of pseudopregnant rats on day 1 of diestrus is followed by ovulation in about 6 days; the subsequent cycle is a pseudopregnant one in only 20% of cases. 2) Castration, ovarian autotransplantation, and pituitary homotransplantation of cyclers, on day 1 of diestrus, is also followed by ovulation in about 6 days, but the subsequent cycle is a pseudopregnant one in 80% of cases. 3) Castration and ovarian autotransplantation, on day 2 postpartum, of rats suckling a 2-pup litter is followed by ovulation in about 7 days, and in 60% of cases the subsequent cycle is a pseudopregnant one (4). In all three experiments viable luteal tissue was absent in the interval between operation and the first postoperative estrus, for the corpora lutea in these ovarian transplants do not survive (21). The operative procedures also affected the pituitary's ability to secrete FTH approximately equally in each experiment, since neither LTH nor the suckling of a 2-pup litter appreciably suppresses FTH secretion or the ovarian response to FTH (4). In the 2nd experiment LTH was secreted by the homotransplanted pituitary (22), and in the 3rd experiment it was maintained by suckling (4). This secretion of LTH was undoubtedly responsible for the high frequency of spontaneous pseudopregnancies following the first postoperative ovulation. It is therefore very probable that a failure of LTH secretion in the first experiment accounted for the low incidence of spontaneous pseudopregnancies. Thus, the removal of the corpora lutea must have led to the cessation of LTH secretion. Together with the inability of progesterone to induce regression of the corpus luteum, these findings certainly show that progesterone does not inhibit the secretion of LTH, and in fact, suggest that progesterone may promote LTH secretion.

The latter possibility is not only consistent with other facts (4), but also with the evidence, summarized elsewhere (4) that supports the theory that LTH secretion is chronically inhibited by a CNS influence (2, 3), and is promoted by agents that prevent this influence from affecting the pituitary. Progesterone could be the agent that suppresses the activity of the CNS inhibiting influence over LTH, during pseudopregnancy, and thus maintains LTH secretion. This postulation of the progesterone-LTH system as one of "positive feedback," is in accord with other evidence that progesterone can maintain LTH secretion (4), and is remarkably useful in explaining the cyclic secretion of LTH, and the ephemerality of the corpus luteum, at least in the rat. The detailed consideration of these phenomena is the subject of a paper in preparation, but has already been briefly outlined elsewhere (4).

The continued growth of the intact rat's corpora lutea under the influence of exogenous progesterone (23, 24) (Table 1) is not only consistent with this interpretation, but it cannot be fitted with the concept that progesterone inhibits LTH secretion. When luteal regression occurs, therefore, in association with progesterone treatment, as may be the case in pregnant pigs and sheep (25), and even in the rat if the treatment is prolonged (26), it more probably suggests the effect of factors that interfere with the luteal response to LTH than it does the absence of LTH. Luteal regression in the rat in fact always occurs, *in spite of the continued secretion of LTH*, as long as the pituitary retains its ability to secrete FTH (4). The possible effect of the latter on luteal viability is also discussed elsewhere (4).

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THE EFFECT OF CORTISONE, HYDROCORTISONE, AND 9α-FLUORO-16α-HYDROXY-Δ¹-HYDROCORTISONE ON THE ACTION OF DESOXYCORTICOSTERONE AND ALDOSTERONE WITH RESPECT TO ELECTROLYTE EXCRETION

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ABSTRACT

The influence of cortisone and hydrocortisone on the action of desoxycorticosterone and aldosterone on sodium and potassium excretion was investigated in adrenalectomized rats. The results showed that with increasing doses of cortisone and hydrocortisone an antagonistic action to the sodium retaining effect of desoxycorticosterone and aldosterone occurred. 9α-fluoro-16α-hydroxy-Δ¹-hydrocortisone, similarly, had a marked antagonistic action at much lower dose levels. The steroids cortisone, hydrocortisone, 9α-fluoro-16α-hydroxy-Δ¹-hydrocortisone, and the steroids desoxycorticosterone and aldosterone, each facilitated potassium excretion in adrenalectomized rats; the simultaneous administration of any one of the former with either of the latter produced an increased effect on potassium excretion.

THE adrenal cortex secretes a number of steroid hormones which affect sodium excretion to a varying degree. The synergistic or antagonistic action of various adrenal cortical hormones on the absorption, distribution, and excretion of electrolytes and their interplay with each other are not well understood. Soffer *et al.* (1, 2) have demonstrated that the amount of sodium chloride excreted after intravenous injection of normal saline was decreased by the prior intramuscular injection of DCA in normal individuals, whereas it was increased by the same amount of DCA under the same condition in patients with Cushing's syndrome. Woodbury *et al.* (3) found that chronic administration of DCA to intact rats increased the concentration of plasma sodium. When ACTH was used concomitantly with DCA, the plasma sodium concentration was restored to normal, whereas the decreased plasma potassium, chloride and magnesium concentration found after chronic administration of DCA was unaffected by ACTH. More recently, Selye (4) has studied the anticortisol action of aldosterone from the morphological point of view and has demonstrated that the concurrent administration of aldosterone and hydrocortisone in the ratio 1 to 8 inhibits all the morphological action of the latter.

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The modified response to DCA after intravenous saline administration in Cushing's syndrome and the lowering effect of ACTH on hypernatremia induced by DCA in intact rats, may be interpreted as an antagonism between the steroids cortisone, hydrocortisone, corticosterone, and the steroids desoxycorticosterone and aldosterone.

In order to study the effect of cortisone, hydrocortisone, and 9α-fluoro-16α-hydroxy-Δ^1-hydrocortisone on the action of desoxycorticosterone and aldosterone on electrolyte excretion, the following experiments were undertaken.

METHOD

Mature male hooded rats, weighing 170 to 180 gm., were used in all experiments. The rats were adapted to a constant temperature (78° F) and humidity (45%) for 24 hours before adrenalectomy and remained on a diet of Purina fox chow and tap water. Bilateral adrenalectomy was performed by the procedure described by Ingle and Griffith (5). Following the operation the rats were given a solution of 1% saline and 5% glucose and were fed the same diet. The rats were used on the third day following adrenalectomy. Fifteen hours before the test, the saline solution was removed and replaced by a 5% glucose solution. Food and drinking fluid were removed one hour before the injection of test substances, which were given at 9 A.M. The rats in the test groups each received subcutaneously 0.1 cc. of 95% ethanol containing the various steroids and 3.5 cc. of distilled water intraperitoneally. The rats in the control group also received 0.1 cc. of ethanol subcutaneously and the same amount of distilled water intraperitoneally as those in the test groups. The animals were put into individual metabolic cages. Urine was collected at different time intervals as indicated in the experiments. At the end of the collection period, inhalation of ether was used to stimulate emptying of the bladder. The funnels of the metabolic cages were washed with 20 cc. of distilled water at the end of the collection of urine for more accurate transfer. Variation in timing was minimized by taking each rat in the same order for all procedures.

In the first experiment shown in Table 1, the rats were allowed to take excess distilled water from the bottle attached to the outside of the metabolic cages. Since little or no fluid was drunk during the experiments, additional drinking fluid was omitted in subsequent experiments.

The sodium and potassium content of the urine was measured by flame photometry using an Internal Lithium Standard (6).

RESULTS

Effect of Cortisone on the Action of DCA

Adrenalectomized rats were subdivided into five groups. There were 12 rats in each group. The rats in Group 1 served as controls. Those in Group 2 received 3 μg. of DCA in 0.1 cc. of 95% ethanol subcutaneously, and those in Groups 3, 4, and 5 received 3 μg. of DCA plus 12, 75, and 120 μg. of cortisone, respectively. Urine samples were collected at intervals of 2 hours. Results are listed in Table 1. Under the condition of this assay, 3 μg. of DCA produced a marked sodium retention, which lasted over 10 hours. When 12 μg. of cortisone was given with 3 μg. of DCA, a greater sodium retention was observed. When the cortisone was increased to 75 μg., the sodium excretion was approximately the same as that of the rats receiving

TABLE 1. THE EFFECT OF DCA AND CORTISONE ON ELECTROLYTE EXCRETION IN ADRENALECTOMIZED RATS

12 rats in each group	Hours				
	2	4	6	8	10
	Urine excretion cc./rat/2 hours Mean ± S.D.				
1. Control	1.08 ±0.48	1.06 ±0.71	1.13 ±0.66 *3.27 ±0.82 (100%)	1.72 ±0.81	1.29 ±0.27
2. DCA 3 μg.	0.57 ±0.23	0.57 ±0.30	1.00 ±0.10 *2.14 ±0.55 (65%)	1.47 ±0.27	1.15 ±0.53
3. DCA 3 μg. Cortisone 12 μg.	0.48 ±0.18	0.39 ±0.15	1.12 ±0.32 *2.09 ±0.43 (63%)	1.28 ±0.53	1.26 ±0.35
4. DCA 3 μg. Cortisone 75 μg.	0.62 ±0.42	1.00 ±0.31	1.58 ±0.44 *3.20 ±0.62 (97%)	1.29 ±0.84	1.10 ±0.29
5. DCA 3 μg. Cortisone 120 μg.	1.10 ±0.61	1.33 ±0.45	1.40 ±0.38 *3.83 ±0.97 (117%)	1.25 ±0.73	1.51 ±0.53
	Na excretion meq./rat/2 hours Mean ± S.D.				
1. Control	0.064 ± 0.006	0.065 ± 0.015	0.085 ± 0.025 *0.214 ± 0.013 (100%)	0.128 ± 0.032	0.126 ± 0.025
2. DCA 3 μg.	0.038 ± 0.003	0.020 ± 0.006	0.047 ± 0.009 *0.105 ± 0.006 (49%)	0.089 ± 0.008	0.089 ± 0.024
3. DCA 3 μg. Cortisone 12 μg.	0.020 ± 0.010	0.010 ± 0.003	0.031 ± 0.008 *0.061 ± 0.012 (28%)	0.050 ± 0.009	0.083 ± 0.003
4. DCA 3 μg. Cortisone 75 μg.	0.036 ± 0.017	0.041 ± 0.003	0.040 ± 0.008 *0.117 ± 0.026 (54%)	0.063 ± 0.017	0.077 ± 0.014
5. DCA 3 μg. Cortisone 120 μg.	0.052 ± 0.017	0.052 ± 0.004	0.095 ± 0.008 *0.199 ± 0.024 (94%)	0.112 ± 0.024	0.136 ± 0.020
	K excretion meq./rat/2 hours Mean ± S.D.				
1. Control	0.028 ± 0.002	0.040 ± 0.014	0.058 ± 0.013 *0.126 ± 0.021 (100%)	0.076 ± 0.021	0.064 ± 0.008
2. DCA 3 μg.	0.027 ± 0.002	0.028 ± 0.008	0.069 ± 0.003 *0.124 ± 0.009 (98%)	0.074 ± 0.014	0.060 ± 0.008
3. DCA 3 μg. Cortisone 12 μg.	0.025 ± 0.004	0.031 ± 0.016	0.087 ± 0.002 *0.143 ± 0.010 (115%)	0.089 ± 0.003	0.072 ± 0.011
4. DCA 3 μg. Cortisone 75 μg.	0.027 ± 0.014	0.049 ± 0.009	0.105 ± 0.012 *0.181 ± 0.034 (143%)	0.060 ± 0.014	0.074 ± 0.009
5. DCA 3 μg. Cortisone 120 μg.	0.035 ± 0.009	0.052 ± 0.012	0.099 ± 0.006 *0.186 ± 0.009 (147%)	0.078 ± 0.014	0.084 ± 0.027

* Total excretion over a 6-hour period.
( ) % of the control value.

3 μg. of DCA, except for the 2- to 4-hour period. With 120 μg. of cortisone, a slight diminution of sodium excretion was noted. In the control group the sodium excretion over a six-hour period was 0.214 ±0.013 meq per rat. The DCA treated group excreted 0.105 ±0.006 meq per rat and the groups to which added cortisone was given in amounts of 12, 75, and 120 μg. excreted 0.061 ±0.012, 0.117 ±0.026, and 0.199 ±0.024 meq per rat, respectively. The mean rate of sodium excretion of the DCA treated group of rats over a 6-hour period was 49% of the control group ($P < 0.01$). When DCA plus 12 μg. of cortisone was given, the sodium excreted was 28% of the control value. This sodium retention is significantly greater ($P < 0.01$) than that of the group treated with DCA alone. With 75 μg. of cortisone, the sodium excretion was 54% and with 120 μg. it was 94% of that of the control. The sodium retaining action of 3 μg. of DCA was diminished by 120 μg. of cortisone and this difference was significant statistically ($P < 0.01$, compared with the value of DCA alone).

The rates of excretion of potassium after DCA and cortisone administration are also shown in Table 1. Concomitant use of cortisone and DCA resulted in a greater potassium excretion than that observed with DCA alone. The potassium excretion over a 6-hour period in the control group was 0.126 ± 0.021 meq per rat. The DCA treated group excreted 0.124 ± 0.009 meq per rat ($P > 0.1$) and the groups to which added cortisone was given in amounts of 12, 75, and 120 μg. excreted 0.143 ± 0.010 ($P > 0.1$, compared with the control), 0.181 ± 0.034 ($P = 0.05$), and 0.186 ± 0.009 ($P < 0.01$) meq per rat, respectively.

Effect of Hydrocortisone on the Action of DCA

The second experiment was performed with hydrocortisone and DCA. Six groups of 12 rats were used. Group 1 served as controls and received ethanol, Group 2 received 3 μg. of DCA, Groups 3, 4, and 5 received 3 μg. of DCA plus varying amounts of hydrocortisone in ethanol. The urine was collected over a 7-hour period. The results are shown in Table 2. The sodium excretion of the control group was 0.215 ± 0.065 meq per rat and that of the rats treated with 3 μg. of DCA was 0.053 ± 0.022 meq per rat. The sodium excretion by the rats treated with 15, 100, 200, and 400 μg. of hydrocortisone in addition to 3 μg. of DCA was respectively 0.121 ± 0.005, 0.230 ± 0.075, 0.257 ± 0.045, and 0.352 ± 0.102 meq per rat. In this experiment, 15 μg. of hydrocortisone diminished the sodium retaining action of DCA ($P < 0.01$), and, at a dose level of 100 μg. of hydrocortisone, the sodium retaining action of 3 μg. of DCA was completely inhibited. With 200 and 400 μg. of hydrocortisone, sodium excretion was greater than that of the control group even in the presence of DCA.

The concomitant use of DCA and hydrocortisone, however, resulted in a greater potassium excretion than that obtained with DCA alone. These results are shown in Table 2. The mean potassium excretion of the rats treated with 3 μg. of DCA was 107% of that of the control ($P > 0.1$). The addition of hydrocortisone in amounts of 15, 100, 200, and 400 μg. resulted in an increased potassium excretion. Potassium excretion was respectively 129, 223, 266, and 351% of that of the control group ($P < 0.01$ in each case).

TABLE 2. THE EFFECT OF DCA AND HYDROCORTISONE ON ELECTROLYTE EXCRETION IN ADRENALECTOMIZED RATS

12 rats in each group	Urine cc./rat Mean ± S.D.	Na meq/rat		K meq/rat	
		Mean ± S.D.	% of the control	Mean ± S.D.	% of the control
1. Control	3.81 ± 1.05	0.215 ± 0.065		0.139 ± 0.032	
2. DCA 3 μg.	2.86 ± 0.85	0.053 ± 0.022	24	0.150 ± 0.016	107
3. DCA 3 μg. Hydrocortisone 15 μg.	3.23 ± 0.85	0.121 ± 0.005	56	0.180 ± 0.062	129
4. DCA 3 μg. Hydrocortisone 100 μg.	5.68 ± 1.45	0.230 ± 0.075	107	0.311 ± 0.075	223
5. DCA 3 μg. Hydrocortisone 200 μg.	7.55 ± 2.50	0.257 ± 0.045	119	0.370 ± 0.043	266
6. DCA 3 μg. Hydrocortisone 400 μg.	8.50 ± 3.76	0.352 ± 0.102	163	0.448 ± 0.046	351

Urine collected over a 7-hour period.

TABLE 3. THE EFFECT OF D-1-ALDOSTERONE AND HYDROCORTISONE ON ELECTROLYTE EXCRETION IN ADRENALECTOMIZED RATS

12 rats in each group	Urine cc./rat Mean ± S.D.	Na meq/rat		K meq/rat	
		Mean ± S.D.	% of the control	Mean ± S.D.	% of the control
1. Control	4.70 ± 2.60	0.112 ± 0.024		0.154 ± 0.025	
2. Aldosterone 1 μg.	3.85 ± 2.10	0.046 ± 0.015	41	0.170 ± 0.047	110
3. Aldosterone 1 μg. Hydrocortisone 15 μg.	3.50 ± 1.25	0.039 ± 0.019	34	0.161 ± 0.031	104
4. Aldosterone 1 μg. Hydrocortisone 100 μg.	5.46 ± 1.00	0.083 ± 0.037	74	0.253 ± 0.069	164
5. Aldosterone 1 μg. Hydrocortisone 200 μg.	6.76 ± 1.85	0.096 ± 0.036	85	0.318 ± 0.087	206
6. Aldosterone 1 μg. Hydrocortisone 400 μg.	6.25 ± 2.40	0.119 ± 0.046	106	0.327 ± 0.048	212

Urine collected over a 7-hour period.

Effect of Hydrocortisone on the Action of Aldosterone

The third experiment was performed with hydrocortisone and aldosterone. The rats of the control group and of the test groups were treated as described in the second experiment. The results are shown in Table 3. The administration of 1 μg. of d-l-aldosterone caused a marked sodium retention compared with that of the control. When 15 μg. of hydrocortisone was given in addition to the aldosterone, more sodium retention occurred, but this difference was not significant ($P > 0.1$). With higher doses 100 and 200 μg. of hydrocortisone added to original dose of 1 μg. of aldosterone, less sodium was retained ($P < 0.01$), and at the level of 400 μg. the sodium retaining action of 1 μg. of d-l-aldosterone was abolished. In contrast to these findings, the concomitant use of hydrocortisone and aldosterone resulted in a more marked potassium excretion than aldosterone used alone. On comparison of the difference between the means of the potassium excretion of the groups treated with aldosterone alone, and with aldosterone and hydrocortisone, P was less than 0.01 in each case except in the group treated with aldosterone and hydrocortisone 15 μg.

The Effect of 9α-fluoro-16α-hydroxy-Δ^1-hydrocortisone on the Action of DCA

The study was carried out in a similar manner using 5 groups of rats and the results are shown in Table 4. Injection of 4 μg. of DCA alone resulted in a marked sodium retention. When 5, 10, or 40 μg. of 9α-fluoro-16α-hydroxy-Δ^1-hydrocortisone were given simultaneously with 4 μg. of DCA the sodium

TABLE 4. THE EFFECT OF 9α-FLUORO-16α-HYDROXY-Δ^1-HYDROCORTISONE (TRIAMCINOLONE) AND DCA ON ELECTROLYTE EXCRETION IN ADRENALECTOMIZED RATS

12 rats in each group	Urine cc./rat Mean ± S.D.	Na meq/rat		K meq/rat	
		Mean ± S.D.	% of the control	Mean ± S.D.	% or the control
1. Control	3.21 ± 0.84	0.108 ± 0.022		0.087 ± 0.035	
2. DCA 4 μg.	2.88 ± 0.69	0.043 ± 0.018	39	0.123 ± 0.013	141
3. DCA 4 μg. Triamcinolone 5 μg.	5.23 ± 0.14	0.135 ± 0.005	125	0.188 ± 0.025	216
4. DCA 4 μg. Triamcinolone 10 μg.	6.17 ± 1.25	0.185 ± 0.036	171	0.259 ± 0.010	297
5. DCA 4 μg. Triamcinolone 40 μg.	7.50 ± 1.80	0.186 ± 0.018	172	0.349 ± 0.026	401

Urine collected over a 6-hour period.

retaining action of DCA was completely inhibited ($P < 0.01$ in each case).

Potassium excretion was, however, enhanced by DCA. Concomitant use of DCA and 9α-fluoro-16α-hydroxy-Δ^1-hydrocortisone resulted in still greater potassium excretion than that observed with DCA alone. On comparison of the difference between the means of the control and treated groups, P was less than 0.01 in each case.

DISCUSSION

Quantitative and qualitative adjustment and interplay between corticosteroids may be an important factor in regulating various biological phenomena. The present studies were undertaken to clarify the question of whether cortisone and hydrocortisone are actually antagonists to aldosterone or desoxycorticosterone with respect to electrolyte metabolism. The results obtained show that rather large amounts of cortisone and hydrocortisone increased sodium excretion even in the presence of DCA or aldosterone, which, in themselves, each caused decreased sodium excretion under the same conditions. Thus, 400 μg. of hydrocortisone nullified the sodium retaining action of 1 μg. of d-l-aldosterone in adrenalectomized rats. At the present time, it is difficult to say whether this effect is brought about by inhibition of tubular reabsorption of sodium or by increasing glomerular filtration rate. Davis and Howell (7) found in the intact dog that a natriuresis accompanied the rise in glomerular filtration rate, induced by cortisone or ACTH. Similarly, Garrod *et al.* (8) demonstrated in the adrenalectomized dog that after cortisone administration there was a clear correlation between filtration rate and sodium output, though the latter was slightly less, and the fraction of filtered sodium reabsorbed slightly higher than at parallel levels of filtration without cortisone. Thus, though cortisone increases sodium reabsorption, this effect is masked by the increased filtration of this ion. Raiz *et al.* (9) also demonstrated that in man single intravenous infusion of hydrocortisone regularly produced an increase in glomerular filtration rate. Moreover, Roberts and Pitts (10), and Garrod *et al.* (8) have shown in the adrenalectomized dog that when cortisone and DCA were given together reabsorption of sodium became complete, despite increased filtration, and that these steroids did not have opposite effect on the renal excretion of sodium. Thorn *et al.* (11), however, reported that patients receiving 5 mg. of DCA per day showed an immediate increase in sodium excretion when 100 mg. of cortisone per day was added. Ross (12) has demonstrated in man that corticosterone or hydrocortisone given simultaneously with aldosterone decreased the sodium retaining effect of aldosterone.

In the present studies in the rats, with increasing doses of cortisone and hydrocortisone, an antagonistic action to the sodium retaining effect of DCA and aldosterone occurred. These natriuretic effects of cortisone and hydrocortisone even in the presence of DCA or aldosterone are most

likely due to the superimposition of the effects of cortisone or hydrocortisone in increasing glomerular filtration rate on their effects on sodium reabsorption by the renal tubules, since most previous studies (7, 8, 9, 13) have indicated that cortisone and hydrocortisone facilitate sodium excretion by increasing the glomerular filtration rate. Dingman *et al.* (14) have, however, reported in man that intravenous administration of hydrocortisone, in small doses, frequently produced a decline in tubular reabsorption of sodium. It is, therefore, still a matter of dispute whether cortisone or hydrocortisone inhibits tubular reabsorption of sodium even in the presence of aldosterone or DCA.

With regard to potassium, various adrenal cortical steroids enhanced the excretion of this electrolyte in the present studies. When the combination of DCA or aldosterone with cortisone or hydrocortisone was given, the action of the two types of corticoids on the renal excretion of potassium was qualitatively similar in contrast to the action on sodium. Besides the renal effect, extrarenal effects, such as an influence on intracellular potassium, may be involved in increased potassium excretion by these steroids.

A synthetic steroid, 9α-fluoro-16α-hydroxy-Δ^1-hydrocortisone, at much lower dose levels than cortisone or hydrocortisone, also antagonized the sodium retaining action of DCA. In the present investigation, 5 μg. of this steroid completely inhibited the sodium retaining action of 4 μg. of DCA. From the present data, it is not possible to determine the mechanism of the natriuretic action of this steroid, which may be due to an increased glomerular filtration rate or to an interference with the tubular reabsorption of sodium. As this steroid has been shown to have a potent hydrocortisone-like activity on carbohydrate metabolism (15), it may act by the same mechanisms as hydrocortisone or cortisone. The presence of the C-16-hydroxyl group in this steroid is of special interest, since potent sodium retaining steroids are deprived of their salt retaining properties by the introduction of a C-16-hydroxyl group (15). Further studies regarding the biological role of 16-hydroxylated steroids would be of interest, because several steroids, having a hydroxyl group at C-16, have been isolated from human urine (16–23).

Acknowledgment

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BIPHASIC EFFECT OF TSH ON THYROIDAL IODIDE COLLECTION IN RATS[1,2]

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ABSTRACT

Assessing the activity of the thyroidal iodide pump by measuring the thyroid/serum radioiodide gradient (T/S), we found the stimulatory effect of TSH to have a latency of from about 8 hours to 12 hours or more. The longer latent periods were observed in rats whose initial T/S was relatively high. The rise in the T/S was preceded by a depression of this ratio, whether hypophysectomized, normal, or triiodothyronine (T_3) treated rats were injected with TSH. This biphasic response was elicited with Armour TSH, lyophilized rat pituitary tissue and Bates' highly purified mouse tumor and bovine TSH preparations; it seems therefore reasonable to attribute it to TSH proper rather than some contaminant(s). The same kind of response was also observed in rats whose thyroids were purged of organic iodine with goitrogens and whose endogenous TSH output was subsequently inhibited by giving T_3 along with the antithyroid drugs; this indicates that the initial depression of the T/S is not due to flooding of the thyroidal iodide pool with stable iodide derived from iodotyrosines through deiodination, nor to inhibition of iodide pump activity by iodothyronines liberated from thyroglobulin by TSH. When organic binding of iodine was not blocked, stimulation of thyroidal I^{131} uptake was observed in hypophysectomized rats 4 hours after the injection of TSH, although in blocked glands of similar animals the T/S was depressed. Unidirectional active iodide clearance by the thyroid (estimated on the basis of clearance during the first 3 minutes following injection of I^{131}) was unchanged in hypophysectomized rats examined 4 hours after the injection of TSH, but the thyroids of such rats lost *in vivo* accumulated radioiodide at a faster rate *in vitro* than did those of untreated hypophysectomized animals. It therefore appears that a relatively early effect of TSH may be an enhancement of the exit rate constant for thyroidal iodide (increased permeability of thyroid cell membranes?). The stimulating influence of TSH on the active uptake of iodide by the rat thyroid, on the other hand, is remarkably slow. Differences between the periods of latency for the effects of TSH on various thyroid functions are discussed.

SEVERAL years ago one of us made the puzzling observation that the initial effect of TSH on the T/S of hypophysectomized rats fed a low iodine diet was to depress this ratio (1). The expected rise in the T/S oc-

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[2] The following abbreviations will be repeatedly used in this paper: TSH = thyrotrophin; T/S = thyroid/serum radioiodide concentration ratio; T/M = thyroid/medium radioiodide concentration ratio; T_3 = 3,5,3′-L-triiodothyronine sodium; PTU = propyl-

curred only later. The purpose of this study was to throw some light on the mechanism whereby TSH lowers the T/S.

MATERIALS AND METHODS

Because of the diversity of the experimental procedures used, this section will only deal with materials and methods common to several experiments. Details regarding individual experiments will be presented in the text of the main section, as footnotes to tables, or as figure captions.

Unless otherwise indicated, young adult male Sprague-Dawley rats were used. Hypophysectomized rats were purchased from the Hormone Assay Laboratories, Chicago, Ill. Only results obtained in rats with testicular and accessory sex gland atrophy and empty sellae, as determined by gross examination, are included in this report. Diets fed were Purina laboratory chow or a low iodine (Remington) diet supplied by Nutritional Biochemicals, Inc., Cleveland, Ohio. After the injection of TSH the rats received only water. The TSH generally used was Armour material containing 0.9 U.S.P. U./mg.[5] If other TSH preparations were employed, this will be mentioned in the text. Blocking of thyroidal binding of iodide as a preliminary to the determination of the T/S was accomplished by the s.c. injection of 6–20 (usually 12) mg. of PTU in alkaline solution. The standard procedure was to inject carrier-free NaI^{131} s.c. 45 minutes later, and to kill the rats 1–1½ hours after that. Departures from this routine will be indicated. The T/S was determined by the method of VanderLaan and Greer (2). All counting of radioactivity was done with a thin mica end-window Geiger-Müller counter. Statistical significance of differences between groups was assessed by Student's t-test. Only those P values which were 0.10 or lower will be presented.

EXPERIMENTAL AND COMMENT

1. *Is the biphasic effect of TSH on the T/S reproducible?*

The experiments which are described in our study permit us to answer this question in the affirmative. The original observation (1) pertained to the effect of 0.9 U. of TSH injected s.c. into hypophysectomized rats maintained on a Remington diet. It showed diminution of the T/S 3–5 hours after TSH, recovery at 7 hours and elevation at 13 hours. Figure 1A demonstrates that a comparable time curve of the T/S was observed following the intravenous administration of 0.9 U. of TSH to similar rats. Figure 1 B shows that even when the T/S was drastically depressed by T_3, a phenomenon analyzed in an earlier paper (3), s.c. administration of 5 U. of TSH further lowered this ratio before raising it. When 5 U. of TSH were injected s.c. into intact rats, a transient diminution of the T/S was again evident. In one experiment (Fig. 1 C) this change was present 4 hours after the injection, but its duration was not determined. In a second experiment

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thiouracil; MMI = methylmercaptoimidazole (Tapazole); s.c. = subcutaneous(-ly); and KRP = Krebs-Ringer-phosphate, pH 7.4.

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[5] TSH was generously supplied by Dr. J. D. Fisher of Armour Pharmaceutical Company, Kankakee, Ill.

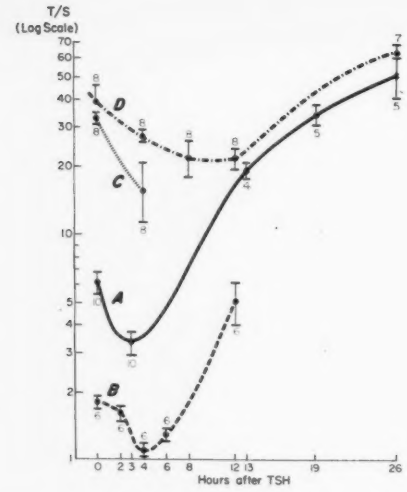

FIG. 1. Time curves of T/S changes after the injection of a single dose of TSH. A: Rats hypophysectomized 20 days earlier and fed the Remington diet for the last 15 days. Nine tenths U. of TSH injected intravenously in 0.5 ml. of 0.9% saline. B: Rats fed Purina and injected s.c. with 5 μg. of T_3 daily for 25 days. Five U. of TSH given s.c. C and D: Untreated rats on Purina, 5 U. of TSH injected s.c. Vertical lines: standard errors. Adjacent numbers indicate number of rats in group. P values: A—0 hour vs. others groups <0.01; B—0 hour vs. 4, 6 and 12 hours <0.01; C—0 hour vs. 4 hours 0.01–0.02; D—0 hour vs. 4 hours 0.08, 0 hour vs. 8 hours 0.03–0.05, 0 hour vs. 12 and 26 hours <0.01.

(Fig. 1 D) lowering of the T/S was most significant 12 hours after TSH administration, i.e., at a time when in hypophysectomized or T_3 treated animals the stimulatory effect was already clear-cut. Further experiments in which the biphasic response of the T/S to TSH was observed will be presented below.

2. *Is the transient depression of the T/S due to TSH proper?*

Since pure TSH is not available, this question cannot be considered finally settled. But the diphasic response of the T/S was obtained with several TSH-containing preparations, ranging from lyophilized anterior pituitary lobes of old male Long-Evans rats, which must have contained many or all of the other pars distalis hormones, to Bates' highly purified beef TSH, which is practically devoid of hormonally active contaminants, and his mouse pituitary tumor TSH, which is prepared from starting material containing no hormones besides TSH[6] (Table 1). This rules out any other pituitary hormone (although not non-hormonal contaminants of TSH) as the agent responsible for the transient depression of the T/S, and makes it permissible to assume, pending the emergence of evidence to the contrary, that we are dealing with an effect of TSH *per se*.

Because even with the administration of large doses of TSH the drop in the T/S was seldom much greater than 50% (see also experiments to be discussed), we did not consider it worthwhile at this time to study dose-effect relationships in detail. If rat pituitary powder contains 0.1 U. of TSH/mg. (4), the rats in group IV of Table 1 received less than 0.2 U. The

[6] We are indebted to Dr. R. W. Bates of the NIH, Bethesda, Md. for samples of his TSH preparations.

TABLE 1. EARLY AND LATE EFFECTS OF VARIOUS TSH-CONTAINING PREPARATIONS (SUBCUTANEOUS INJECTION) ON THE T/S OF RATS 13 DAYS (EXPTS. I–III) OR 28 DAYS (EXPT. IV) AFTER HYPOPHYSECTOMY. REMINGTON DIET WAS FED DURING THE LAST 8 DAYS (EXPT. I–III) OR 17 DAYS (EXPT. IV)

Expt. No.	Preparation	Dose/rat	Uninjected controls	4	24	P	
				Hours after TSH		4 hrs. vs. cont.	24 hrs. vs. cont.
I	Armour TSH (0.9 U./mg.)	0.9 U.		3.4±0.5 (8)	90.4±10.3 (4)	0.08	<0.01
II	Mouse tumor TSH (2.3 U./mg.)	0.9 U.	5.3±0.7 (13)	2.6±0.4 (6)	127.9±23.1 (4)	0.03	<0.01
III	Purified beef TSH (13 U./mg.)	0.9 U.		2.9±0.2 (8)	67.0±10.8 (4)	0.03	<0.01
IV	Lyophilized rat anterior lobe	1.69 mg.	5.9±0.8 (8)	3.4±0.3 (8)	103.4± 6.7 (5)	0.02	<0.01

All T/S values mean±S.E. Number of rats in parentheses. Potencies of TSH preparations as reported by the company or Dr. Bates.

minimum effective dose must then be lower than this amount if the recipients are hypophysectomized rats fed a low iodine diet. The possibility that rat TSH may be more effective in rats than TSH from other species must be kept in mind. The potency of the TSH preparations which we used was determined by assays in chicks, to which, of course, any mammalian TSH is a foreign protein.

3. *Is the depression of the T/S due to flooding of the thyroidal iodide pool with stable iodide or inhibition of the iodide pump by free iodothyronines?*

It was hypothesized (1) that a transitional drop in the T/S may be due to swamping of the thyroidal iodide pool with stable iodide released by iodotyrosines that are set free from thyroglobulin under the effect of TSH. Consideration was also given to the alternative idea that free iodothyronines resulting from the TSH-accelerated hydrolysis of thyroglobulin might be responsible for the decline of the T/S (1). There is evidence for an intrathyroidal inhibitor of thyroidal iodide collection, and several observations suggest that this inhibitor is a compound (or a group of compounds) which contains iodine in organic form (cf. 5). Both flooding of the iodide pool with stable iodide and inhibition of iodide transport by free iodothyronines could be dismissed as an important cause of the T/S depression after TSH if this effect could also be obtained in glands purged of organic iodine by means of antithyroid drugs.

First we tried animals made goitrous (thyroid weights averaging over 50 mg.) by the daily s.c. injection of 20 mg. of PTU for 22 days (diet: Purina chow). Four hours after the s.c. injection of 5 U. of TSH the T/S of 7 such rats was 119.6±4.7 (S.E.) compared with 123.4±13.1 in 8 controls not given TSH. In further experiments we combined depletion of thyroidal organic iodine stores with inhibition of TSH output. For this purpose rats were given goitrogens and then, while the antithyroid regimen was continued, injected with T_3 (6). In the first experiment (Fig. 2A) rats fed

Purina were given 0.1% MMI and 1% glucose in their drinking water for 19 days. (Sampling of the group showed that this caused thyroid enlargement to 30 mg. average weight.) Then this goitrogenic regimen was combined with the daily s.c. injection of 5 μg. of T_3 during the following 15 days. At the end of this period 5 U. of TSH were injected s.c. Thyroid weights at this time averaged 11 mg. The T/S of these rats was not as low as that of rats treated with thiouracil followed by thiouracil and T_3 (3). The response of the T/S to injected TSH was quite similar to that seen in hypophysectomized rats (1, Fig. 1A of this paper). In the other 2 experiments (Fig. 2B and C) rats maintained on Purina were subjected to a more

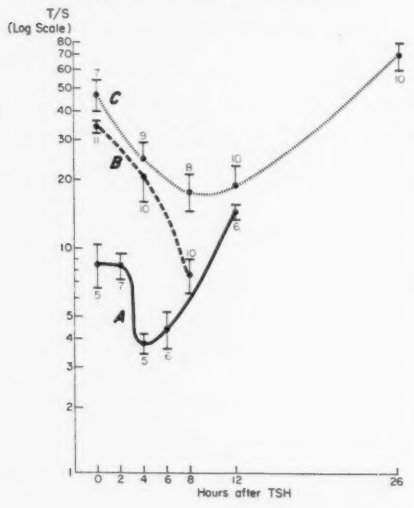

FIG. 2. Time curves of T/S changes after a single s.c. injection of 5 U. of TSH. A: Rats fed MMI and then also injected with T_3. B and C: Rats injected with PTU and then with PTU plus T_3. See text for details regarding dosages and schedules. Vertical lines: standard errors. Adjacent numbers indicate number of rats in group. P values: A—0 hour vs. 4 hours 0.03–0.05, 0 hour vs. 6 hours 0.06–0.07, 0 hour vs. 12 hours 0.01–0.02, 2 hours vs. 4 hours <0.01, 2 hours vs. 6 hours 0.03, 2 hours vs. 12 hours <0.01. B—0 hour vs. 4 hours 0.02, 0 hour vs. 8 hours <0.01. C—0 hour vs. 4 hours 0.01–0.02, 0 hour vs. 8 and 12 hours <0.01, 0 hour vs. 26 hours 0.10.

potent goitrogenic regimen, viz., the s.c. injection of 20 mg. of PTU in suspension form daily for 19 days. This treatment results in goiters weighing, on the average, around 50 mg. Subsequently, while the PTU injections were continued, 10 μg. of T_3 daily were also injected s.c. for 9 days. Thyroid weights at necropsy averaged 24 mg. The T/S was as high as or higher than that of normal rats (compare Fig. 2B and C with Fig. 1C and D), and its response to 5 U. of TSH s.c. was different from that shown in Fig. 2A but similar to that of intact animals: the initial depression reached its nadir slowly and the rise was delayed. In fact, 26 hours after the injection of TSH (Fig. 2C) the T/S was not yet significantly higher than the initial value. This is in sharp contrast with the 10–20-fold increase which was seen in hypophysectomized rats 24 hours after the administration of much smaller doses of TSH (Table 1). The reason for this difference remains to be elucidated. Further studies will also have to be conducted to determine whether the generalization can be made that when the T/S is relatively high, its response to TSH is drawn out.

The experiments whose results are summarized in Figure 2 clearly show that ample stores of organic iodine in the thyroid are not a prerequisite for the transitory depression of the T/S by TSH, and that flooding of the thyroidal iodide pool with stable iodide derived from iodotyrosines or inhibition of the iodide pump by free iodothyronines therefore cannot play an essential role in the fall of the T/S which follows TSH administration.

4. *Kinetic considerations regarding the lowered T/S after TSH*

According to Wollman and Reed (7) the T/S, measured when isotopic equilibrium is achieved, equals $C/m_T/K_{TB}$, where C/m_T stands for the unidirectional clearance of radioiodide per mg. thyroid, and K_{TB} is the exit rate constant for thyroidal radioiodide. A low T/S not representative of the stable iodide gradient between thyroid and serum is encountered if it is determined at a time when it still falls grossly short of its equilibrium value. We therefore considered the possibility that equilibration of radioiodide between thyroid and blood is slowed down during the period after TSH administration when the T/S is depressed. However, this depression was as pronounced when $3\frac{1}{2}$ hours were allowed for the equilibration of radioiodide (Table 3) as when the T/S was measured 1–$1\frac{1}{2}$ hours after the injection of I^{131}, which was our practice in other experiments.[7]

According to Wollman and Reed's formula for the equilibrium T/S, a drop in the T/S could be due to a diminished C/m_T, and increased K_{TB}, an enhancement of both, with that of the K_{TB} being greater, or a decrease in both, with that of the C/m_T being more marked. The C/m_T can be estimated at short times after I^{131} injection, when the amount of radioiodide that is returned from the thyroid to the blood is still small enough to be negligible (7). If the function of an iodide pump with low activity is to be thus tested, a correction must be made for the amount of radioiodide that has entered the thyroid by diffusion—a negligible quantity in conditions of more vigorous thyroid pump activity such as those studied by Wollman

[7] Wollman and Reed (7) found in rats with widely varying thyroidal iodide pump function that 95% equilibration of radioiodide between thyroid and blood was achieved in 60 minutes or less after its intraperitoneal injection. However, they did not examine rats with an average T/S of less than 25, and did not try s.c. injections. We therefore investigated groups of normal, hypophysectomized and T_3 treated rats 20, 40, 60, 90, 120 and 240 minutes after s.c. administration of I^{131}. We found that the T/S at 40 minutes was not significantly lower than at 240 minutes (30.7 ± 3.6 S.E. vs. 30.1 ± 2.3 in normal rats on Purina, 5.0 ± 0.7 vs. 6.1 ± 1.1 in hypophysectomized rats 19 days after the operation which were also fed Purina, and 1.6 ± 0.2 vs. 1.7 ± 0.2 in rats injected for 22 days with 5 μg. of T_3 daily and fed Purina for 10 days, followed by the Remington diet for 12 days during which 0.1% MMI was also given in the drinking water). We don't know of any condition in which the T/S of rats, as determined by the routine method, i.e., 60–90 minutes after the s.c. injection of I^{131}, is not reasonably close to the equilibrium T/S, which could only be attained if blood levels of radioiodide were prevented from falling (7).

and Reed. The appropriately corrected C/m_T may not be reliable in absolute terms (the assumption is made in its calculation that injected I^{131} instantaneously enters the blood, which is probably not true), but we believe that it is a valid index of the magnitude of the actual C/m_T in various conditions, and can therefore be safely used when groups of animals are compared.

The experimental procedure was the following. Two groups of 10 rats each received the Remington diet for 22 days, starting 5 days after hypophysectomy. The rats in one group were injected s.c. with 5 U. of TSH, those in the other served as controls. All rats were given 12 mg. of PTU s.c. 45–80 minutes before the s.c. injection of 400 μc. of I^{131}. (The I^{131} was injected exactly 4 hours after the TSH in the group treated with this hormone.) One and one-half minutes after the administration of I^{131} ether anesthesia was started; $1\frac{1}{2}$ minutes later blood was withdrawn from the aorta. The thyroid and a piece of submaxillary gland were promptly dissected, weighed and counted. The plasma clearance of radioiodide by 1 mg. of the submaxillary gland, an organ which in the rat lacks an iodide pump and has an iodide space of the same order of magnitude as that of the ClO_4^--blocked thyroid (8), was subtracted from the clearance by 1 mg. of thyroid tissue. We assumed that TSH had no effect on diffusion of iodide into the thyroid, since the iodide diffusion space of the gland is unaffected by the degree of thyrotrophic stimulation (8). The plasma radioiodide level used in the calculation of the clearance was one-half the value found 3 minutes after the injection of I^{131}.

The active unidirectional radioiodide clearance of the thyroid estimated by the procedure described was 0.26 ± 0.04 (S.E.) μl./mg./min. in the controls and 0.25 ± 0.03 μl./mg./min. 4 hours after TSH injection.[8] It seems therefore that C/m_T, which in several conditions is a better measure of thyrotrophic action on the iodide pump than is the T/S (7), is not lowered at a time after the administration of TSH when the T/S is depressed, nor is it, however, enhanced. According to Wollman and Reed's formula the reduction in the T/S should then be due to an increased K_{TB}.

In vivo estimation of the K_{TB} in thyroids with low iodide concentrating ability is technically difficult and unreliable, and was therefore not attempted. However, we did investigate the *in vitro* loss of *in vivo* accumulated radioiodide from the thyroids of hypophysectomized rats, and the effects of injected TSH thereupon. Entire thyroid lobes were used to minimize traumatization. In interpreting the findings thus obtained one must keep in mind that when a lobe loses radioiodide to the surrounding medium, the rate of this process may be limited by factors other than those which determine the exit rate of iodide from the thyroid cell into adjacent capillaries.

Two groups of 6 rats each were hypophysectomized and fed the Remington diet for 24 days, starting 6 days postoperatively. The rats of one group were injected s.c. with 5 U. of TSH; those of the other group served as controls. All rats received 12 mg. PTU

[8] Unidirectional clearance values not corrected for radioiodide that had entered the thyroid by diffusion also failed to show a significant difference: they were 0.41 ± 0.04/mg./min. for the controls and 0.43 ± 0.02 for the TSH treated rats.

s.c. 45 minutes before the s.c. injection of 150 μc. of I^{131}. Sixty to ninety minutes after the injection of I^{131} (but exactly 4 hours after the administration of TSH in the group treated with this substance) the rats were killed with ether, their thyroid lobes dissected out separately, and each thyroid transferred rapidly to a flask containing 1 ml. of KRP with 10^{-3}M MMI. The thyroids were incubated at 37° C in a Dubnoff shaker (110 cycles per minute) for 10 minutes. They were carefully blotted after removal from the medium and digested in 0.5–1.1 ml. of 25% NaOH. Suitable aliquots of the medium and the thyroid digests were counted.

The results of the experiment are shown in Table 2. The final average T/M was so high as to suggest that re-entry of radioiodide from the medium into the thyroid was still negligible compared with the exit, i.e., that

TABLE 2. *In vitro* RELEASE OF *in vivo* ACCUMULATED RADIOIODIDE FROM THE THYROIDS OF HYPOPHYSECTOMIZED TSH-TREATED OR UNTREATED RATS

Group	No. rats	Initial counts per thyroid	% activity released in 10 min.	Final avg. T/M
Hypex, TSH	6	5,635 ± 330*	61.5 ± 3.7	6.6
Hypex, no TSH	6	13,722 ± 3,280†	48.0 ± 4.3†	11.6

* Mean ± S.E.
† $P < 0.05$.

equilibrium between thyroid and medium was not yet approached (cf. Tables 2 and 4). The greater initial activity of the thyroids from untreated rats was probably the reflection of the expected higher T/S in these animals. The percentage loss of radioiodide from the thyroids of the TSH treated rats was greater than the loss from control glands. This may indicate that the former indeed had a higher K_{TB} *in vivo*, as postulated from the measurement of the T/S and the estimation of the C/m_T. Wollman and Reed (7) contemplated whether "the decrease of the T/S to half its initial value 4 hours after TSH injection in hypophysectomized rats is due to a rapid rise in the [iodide] exit rate constant from its value in hypophysectomized rats to the value observed for normal rats." Our results are compatible with this idea.

Because of the use of *in vivo* and *in vitro* techniques in our kinetic studies, and since their interpretation involves a number of assumptions, any conclusion drawn from them must be considered tentative. We have not attempted to analyze the kinetics of the depressed T/S in normal rats given TSH, which may not be the same as in hypophysectomized rats.

If iodide leaves the thyroid cell by diffusion (and there is no reason to believe that it does not), the increase in the exit rate of thyroidal iodide which occurs at least *in vitro* after the injection of TSH may indicate an enhanced permeability of thyroid cell membranes. Hormonal influences on cell permeability have received considerable attention (9). It would be reckless to suggest without good supporting evidence that the primary thyroidal effect of TSH is on cell permeability. However, if TSH leads to enhanced

permeability of the thyroid cell not only to iodide which leaves it but also to substances which enter it by diffusion, this quite probably facilitates the thyroid anabolic functions of TSH even though it may not initiate them.

5. *Comparison of the early effect of TSH in blocked and non-blocked thyroids*

If an early action of TSH on thyroidal iodide collection is to promote discharge of iodide, such an effect should be at least partly obscured by binding of iodide, since this reduces the exit of iodide as such from the thyroid, even if it is now believed not to abolish it entirely (10). In this section we report an experiment, the results of which are consistent with the above

TABLE 3. COMPARISON OF THE EFFECT OF TSH ON THE T/S OF BLOCKED THYROIDS AND THE I^{131} UPTAKE OF NON-BLOCKED GLANDS (HYPOPHYSECTOMIZED RATS 14–15 DAYS AFTER THE OPERATION, PURINA DIET)

Treatment	A. Binding blocked		B. Binding not blocked		
	No. rats	T/S	No. rats	Thyroid/serum I^{131} conc.	% dose I^{131} in 100 mg. thyroid
No TSH	10	5.0±0.4*	10	7.5±0.7	0.61±0.08
TSH	10	2.1±0.1†	9	14.7±2.0†	1.47±0.28‡

* Mean ±S.E.
† $P<0.01$.
‡ $P=0.02$.

Schedules:

A

0 min. 20 mg. PTU
15 min. 5 U. TSH
45 min. I^{131}
255 min. rats killed
(all s.c.)

B

0 min. 5 U. TSH
15 min. I^{131}
240 min. rats killed
(both s.c.)

premise. We compared the effect of TSH on the T/S of hypophysectomized rats with its influence on I^{131} uptake by the thyroids of similar rats in which organic binding of iodide was permitted to proceed. Results and experimental details are presented in Table 3. It is evident that the T/S showed the decline which was consistently observed in hypophysectomized rats 4 hours after the injection of TSH, but that uptake of I^{131} by the non-blocked thyroid during the $3\frac{3}{4}$ hour period which started 15 minutes after the administration of TSH was significantly augmented. Any concomitant increase in the rate of loss of radioiodide from the thyroid that may have occurred was overshadowed.

It appears that organic binding of iodine by the rat thyroid is stimulated by TSH at a time when iodide clearance in the absence of binding is not yet affected (see previous section) and the T/S is lowered. This is in agreement with the idea that thyroidal iodide concentrating is not the rate limiting step in thyroid hormone biogenesis (10, 11), unless it is severely impaired by SCN^- or ClO_4^-. It is possible that stimulation of thyroidal organification of iodide, by depleting the intrathyroidal iodide pool (which has been shown to exist in non-blocked glands (11)), secondarily promotes

iodide transport into the gland even during the early period after TSH administration when enhancing effects of TSH on the iodide pump "chemically dissected" with PTU are not yet evident. Our results fail to throw any light on this point.

6. *Attempts to increase the T/S with TSH in vitro*

Our observations regarding the considerable latency with which TSH raises the T/S of rats *in vivo* are in contrast with those of Bakke and Lawrence (12) who were able to increase the iodide uptake of PTU-blocked beef thyroid slices by incubating them with TSH *in vitro* for only 2 or 4 hours. We tried to achieve results similar to those of Bakke and Lawrence by exposing rat thyroids *in vitro* to TSH.

The donors were 4 groups of rats: a) 32 Sprague-Dawley rats 80–82 days after hypophysectomy; b) 16 old normal Long-Evans rats; c) 6 Sprague-Dawley rats injected

TABLE 4. EFFECT OF TSH IN THE MEDIUM ON *in vitro* UPTAKE OF RADIOIODIDE BY RAT THYROID TISSUE

Donor rats			No TSH		TSH		P
Strain	No.	Condition or treatment	No. flasks	T/M	No. flasks	T/M	
Sprague-Dawley	32	Hypex	4	1.5±0.2*	4	1.3±0.1	n.s.†
Long-Evans	16	Normal	4	4.2±0.3	4	2.7±0.3	0.02
Sprague-Dawley	6	PTU	5	10.3±0.6	6	9.3±1.0	n.s.
Sprague-Dawley	12	PTU, PTU+T_3	6	3.3±0.3	6	4.0±0.7	n.s.

* Mean ±S.E.
† Not significant.

s.c. with 20 mg. of PTU in suspension form daily for 13–17 days, and d) 12 rats treated the same way with PTU for 27 days and injected s.c. with 10 μg. of T_3 daily for the last 8 days of this period. All rats were fed Purina. They were killed with ether, and the rapidly removed thyroids cut into pieces weighing about 2–3 mg. which were pooled in ice-cold KRP and then transferred to flasks containing 4 ml. of KRP with 10^{-3} M MMI, 0.5 or 1 μc. of I^{131}, and either 20 mU. of TSH/ml. or no TSH. The flasks were kept for 2 hours at 37° C in a Dubnoff shaker (110 cycles per minute). At the end of this period the thyroid tissue was removed from each flask, blotted and weighed. The weight of tissue per flask averaged around 20 mg. Both thyroid tissue and aliquots of the medium were counted, and the T/M was recorded (Table 4).

The T/M values for the various experimental groups reflected differences which must have existed in thyroidal iodide pump activity among the donor rats, but were considerably lower than the T/S of comparable rats was found to be in this study and others. In none of the experiments did TSH in the medium elevate the T/M. In the case of thyroids from normal rats it actually appeared to depress it. It may be that stable iodide released from the thyroid tissue into the medium was in part responsible for this. The thyroids of goitrogen treated animals, however, must have been severely depleted of organic iodine. In the experiments in which such glands were used, loading of the medium with iodide derived from thyroglobulin

could not have masked a stimulating effect of TSH on the iodide pump *in vitro*. It is possible that the thyroids from rats treated with PTU only were not responsive to TSH because they were already maximally stimulated, but this could not have been the case when the donors were rats whose thyroids were depressed with T_3 during the later period of their treatment with PTU. Our results agree with the *in vivo* observations in rats which failed to demonstrate any enhancement of thyroidal iodide pump activity for several hours after TSH injection. The difference between our findings and those of Bakke and Lawrence (12) may be simply due to technical reasons. On the other hand, it may be significant that they applied beef TSH to beef thyroid tissue whereas we exposed rat thyroids to bovine TSH. Perhaps this is an important difference *in vitro* even though the pattern of the T/S response is much the same *in vivo* whether beef or rat TSH is given to hypophysectomized rats (Table 1).

7. *Discussion of the latency of the TSH effect on various aspects of thyroid function*

According to the data in this study an elevation of the T/S did not occur in rats for at least 8 hours after the injection of TSH. This agrees with observations made in man (13). An increase in the exit rate of thyroidal iodide appeared to have been responsible for the drop of the T/S which was seen before the T/S rose in response to TSH. Unidirectional clearance of iodide by the thyroid may have risen before the T/S did, but 4 hours after the injection of TSH into hypophysectomized rats an augmentation of unidirectional clearance was not yet demonstrable. On the other hand, binding of I^{131} was already accelerated at this time. Taurog *et al.* (14) have shown a significant shift in the proportion of I^{131}-labelled thyroidal compounds (decreased monoiodotyrosine/diiodotyrosine ratio, increased percentage of thyroxine) 7 hours after the administration of TSH to hypophysectomized rats. Even shorter periods of latency have been reported for other thyroidal effects of TSH. For example, enhanced release of I^{131} from the thyroid was demonstrable by external counting between 2–3 hours following the injection of TSH into rats (15). In dogs whose thyroid vein was cannulated, increased discharge of thyroidal I^{131} in response to TSH had a latency of as little as 15–20 minutes (16). Various facets of the carbohydrate and lipid metabolism of thyroid slices were clearly stimulated *in vitro* by TSH within an hour of incubation, and extrapolation from later observations suggested that the actions of TSH must have been virtually devoid of a latent period (17). In guinea pigs, TSH was reported to increase the number of colloid droplets in the thyroid cell within 15 minutes after its injection (18); in 2 hours it caused growth of thyroid cells and their nuclei (19).

It is clear that the periods of latency for different effects of TSH on the thyroid are remarkably dissimilar. This may indicate that TSH has several points of attack in the thyroid. However, a single thyroidal locus for the

action of TSH is perhaps a more pleasing concept (20, 14). It may be that TSH triggers a series of chemical reactions, and the compounds produced at different times in the course of these events mediate the influence of TSH on individual thyroid responses with widely different latent periods. It is also conceivable that some effects of TSH consist of (direct or indirect) activation of enzymes already present in the thyroid, whereas others (such as the effect on iodide transport into the thyroid) have a considerable latency because they involve the formation of new enzyme (or specific carrier) molecules.

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PARATHYROID HORMONE-LIKE ACTIVITY IN THE SERA OF RATS WITH INDUCED HYPERPARATHYROIDISM

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ABSTRACT

A hyperparathyroid condition was induced in rats by feeding a calcium-deficient, vitamin D-free diet. Serum calcium levels were maintained in the 8 to 11 mg.% range during the twenty-day trial. Parathyroid hormone-like activity was assayed by the rise in serum calcium of thyroparathyroidectomized rats following the injection of sera from the hyperparathyroid rats. Parathyroid hormone-like activity was not assayable until the eighth day of the experiment, although serum calcium levels and histological sections of the parathyroid glands and bone indicated that increased activity of the parathyroid glands had occurred by the sixth day of the dietary regime.

A comparison of the serum calcium rise in the assay animals induced by the injection of standard parathyroid hormone, with that induced by the injection of serum, allowed an estimation of 20 to 64 units of parathyroid hormone equivalence per cc. of serum from calcium-deficient rats. A graded parathyroid hormone-like response was obtained by varying the amount of serum assayed. Parathyroid hormone-like activity was not assayable in 1 cc. of sera from normal rats.

A GREATER portion of the recent work on the parathyroid glands has been devoted to assaying glandular extracts (1, 2, 3, 4), to determining the mode of action of parathyroid hormone (5, 6, 7), and to ascertaining the chemical nature of parathyroid hormone (4, 8, 9, 10). There are only a few reports in the literature of attempts to detect parathyroid hormone in the blood of animals (11, 12), and these were of a qualitative nature.

The purpose of the present study was to biologically assay the sera of hyperparathyroid rats for parathyroid hormone-like activity, in animals sensitive to parathyroid hormone action, and to quantitate the serum activity in terms of a standard parathyroid hormone preparation.

MATERIALS AND METHODS

Induced, or secondary, hyperparathyroidism can result in rats from a low calcium diet, during pregnancy and lactation. A calcium-restricted diet in normal animals is a

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practical means of inducing parathyroid hyperfunction for experimental purposes (13). The calcium-deficient, vitamin D-free diet of Crawford *et al.* (14), with slight modification, was fed *ad lib.* to the test rats. The diet was modified by the substitution of calcium pantothenate for sodium pantothenate and the addition of wool flock, the wool flock preventing the marked diarrhea noticed in rats fed the Crawford Diet in preliminary studies. The modified Crawford Diet, referred to as the C-D diet in this paper, contained 0.001% calcium and 2.4% phosphorus, in contrast to a normal rat diet of 0.8% calcium and 0.4% phosphorus.

The cages, watering tubes, and feed jars were scrubbed with a dilute solution of hydrochloric acid in order to insure that rats on the C-D diet did not have access to calcium deposition on the equipment. Distilled drinking water containing less than 2 ppm of calcium was supplied to the rats on the C-D diet.

The control and thyroparathyroidectomized assay rats were maintained on the nutritionally adequate diet developed by Ullrey and Miller of the Animal Husbandry Department, Michigan State University, and were supplied with tap drinking water. In all other respects the rats were treated in the same manner as the C-D dietary rats.

Ten control male and female Long-Evans Hooded rats were maintained for determination of normal serum calcium, phosphorus and protein determination. Twenty female and 10 male Long-Evans Hooded rats weighing 150 to 200 gm. and 200 to 250 gm., respectively, were placed on the C-D diet. In the initial trial, blood samples were obtained immediately prior to autopsy from each of 2 female rats that were on the C-D diet for 10, 15, 20, 25, and 29 days. In the second trial, blood samples were obtained the day previous to, and immediately prior to, autopsy from 1 male and 1 female rat that that had been on the C-D diet for 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 days. The data from the two trials are considered together.

In order to correlate the serum parathyroid hormone-like activity with the amount of parathyroid gland hypertrophy and with the activity of the circulating parathyroid hormone, blood samples were collected immediately prior to autopsy, and the parathyroid glands and tibias were removed at autopsy for histological analysis. Kidney sections were prepared from rats at representative stages of the dietary regime. Parathyroid and kidney glands were fixed in 10% neutral formalin and processed through a butyl alcohol series, embedded in paraffin, sectioned and stained with hemotoxylin and erosin. Calcium deposition in kidney sections was verified by Van Koss's technique. Tibias were decalcified with "Decal" prior to sectioning.

The assay animals were 55 male Sprague-Dawley rats weighing 115 to 130 gm. They were thyroparathyroidectomized 3 to 4 days prior to the serum assays. There was no attempt made after surgical thyroparathyroidectomy to determine whether accessory parathyroid glands were present. Postoperative rats with serum calcium levels below 8.0 mg.% were considered parathyroidectomized and suitable for assay. Standard parathyroid hormone preparations and blood sera were administered subcutaneously over the lumbar region in 0.5 cc. volumes. A blood sample was drawn from the assay animal before injection of the material to be assayed and again 18 hours later (10).

Blood samples were collected from the orbital sinuses of the rats by the method of Stone (15) with heparinized capillary tubes. The serum was separated as soon as clotting occurred and frozen until assayed. Serum calcium levels were determined on 0.1 cc. of serum by colorimetric titration with E.D.T.A.[3] using ammonium purpurate as an indicator (16). The serum inorganic phosphorus levels were determined by a modification of the molydivanadate method of Simonsen *et al.* (17) on 0.1 cc. of serum. Wechelsbaum's modification of Kingsley's biuret method (18) was employed to determine the total protein content of the serum to be assayed.

[3] Disodium ethylenediaminetetraacetate, a chelating agent sold under the trade name of "Versene" by Versenses Incorporated, Framingham, Massachusetts.

RESULTS

In order to quantitate the serum parathyroid hormone-like activity and to express activity in terms of parathyroid hormone equivalence, standard parathyroid hormone preparations[4] were assayed in 15 thyroparathyroidectomized rats, three rats at each dose level of 10, 15, 20, 40, and 80 U.S.P. units. The results are tabulated in Table 1. Standard statistical analysis of these data demonstrates the slope of the dose-response curve to be $\hat{Y} = -3.64 \pm 3.75X$, where $\hat{Y}$ is the mg.% rise in serum calcium in the assay rat and X is the logarithm of the standard dose injected. It is noted that a

TABLE 1. SERUM CALCIUM RISE IN ASSAY ANIMALS* AFTER INJECTION OF STANDARD PARATHYROID HORMONE

Weight, gm.	Dose, U.S.P. units	Base Ca,† mg.%	Rise in Ca,‡ mg.%	Av. Ca rise, mg.%
101	10	7.8	0.2	
111	10	6.8	0.2	0.13
117	10	7.0	0	
100	15	7.0	0.5	
107	15	6.5	0.5	0.56
130	15	6.3	0.7	
90	20	8.0	0.8	
117	20	7.4	1.6	1.16
125	20	7.8	1.1	
110	40	6.0	3.0	
118	40	6.8	2.0	2.53
124	40	7.7	2.6	
110	80	6.0	4.0	
111	80	7.3	3.5	3.60
117	80	7.5	3.3	

* Thyroparathyroidectomized rats.
† Determined 3 hours prior to parathyroid hormone injections into assay animal.
‡ Determined 18 hours after injection of parathyroid hormone into assay animal.

definite amount of parathyroid hormone is necessary (approximately 9.4 units, calculated from the dose-response curve) to maintain the pre-injection serum calcium level in thyroparathyroidectomized assay rats. Therefore the maintenance of, or even a slight decrease in, the serum calcium level of the assay rat 18 hours from the serum injection is indicative of a positive parathyroid hormone response.

One cc. serum samples from the control rats, containing 9.8 mg.% calcium, were injected into assay rats in order to test the possibility that the calcium content of the serum assayed might be sufficient to cause a rise in

[4] The parathyroid hormone preparation used in this study was "Injection Parathyroid, U.S.P.," generously supplied by the Eli Lilly Company, Indianapolis, Indiana, and contained 100 U.S.P. units per cc.

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the serum calcium of the assay animal. The serum calcium levels of the assay rats continued to decrease during the 18-hour interval between serum calcium determinations, indicating that the calcium content of the serum from the C-D dietary rats would not appreciably influence the assay.

Parathyroid hormone-like activity did not exceed 10 units in the 0.5 cc. serum samples assayed until the test rats had been on the C-D diet for 8 days. The amounts of parathyroid hormone-like activity varied from 20.4 to 64.0 units per cc. of serum from rats on the C-D diet for 8 to 29 days. Although the highest assayable serum activity was from rats on the C-D diet 29 days, there was no clear relationship between the length of time on the

TABLE 2. SERUM CALCIUM RISE IN ASSAY ANIMALS AFTER INJECTION OF GRADED DOSES OF SERUM FROM THE TEST ANIMALS

Days test animals on C-D diet	Dose of serum, cc.	Base Ca,* mg.%	Ca change,† mg.%	Parathyroid hormone equiv., U.S.P. units‡
6	0.25	7.3	−0.7	0
6	0.50	7.5	−0.5	0
6	1.00	7.5	−0.2	0
8	0.60	7.0	0.7	14
8	0.90	7.0	1.0	17
16	0.25	7.8	0.2	11
16	0.50	6.0	0.8	15
16	1.00	7.0	1.5	24
29	0.25	6.6	0.9	16
29	0.50	7.5	1.7	27
29	1.00	7.3	2.5	43

* Determined on thyroparathyroidectomized rats 3 hours prior to injection of test serum
† 18 hours following injection of test sera into assay rat.
‡ In sera of test animal.

C-D diet, that is, the extent of calcium stress, and the amounts of circulating parathyroid hormone-like activity.

The sera from four rats on the C-D diet were assayed by injecting graded doses of each serum sample into the assay rats (Table 2). A graded response was obtained in the assay rats to graded doses of sera from rats with induced hyperparathyroidism. If the serum calcium rise produced in the assay animals, after the injection of graded doses of sera from the C-D dietary rats, is plotted against the logarithm of the dose of serum in cc., a straight line dose-response curve is obtained. The slope of the curve, however, is different from that obtained by assaying standard parathyroid hormone preparation.

The serum calcium levels of the control rats averaged 9.8 mg.%; serum inorganic phosphorus averaged 5.2 mg.%; and the total serum proteins averaged 6.2 mg.%. Serum calcium levels were determined daily on the C-D dietary rats the first 10 days of the experiment, and at approximately two-day intervals to the 29th day. The C-D dietary rats maintained serum calcium levels in the 9 to 11 mg.% range, with the exception of the 5th day of the diet, when the level declined to 8.3 mg.% and between the 12th and

15th day of the diet, when the level declined to between 8.0 and 8.4 mg.% for a 4-day period before returning within the normal range. Serum inorganic phosphorus levels were determined approximately every other day throughout the experiment. Serum inorganic phosphorus increased from the second day of the C-D diet (5.2 mg.%) and continued rising, except for a mild regression between the 15th and 18th days of the experiment. The abrupt rise in serum inorganic phosphorus between the 12th and 15th days, from 5.7 to 7.7 mg.%, coincided with the transient drop in the serum calcium. The total serum protein level remained constant at approximately 6.1 gm.% throughout the experiment.

Histological sections of the parathyroid glands and tibias of rats on the C-D diet demonstrated that parathyroid hypertrophy and discernible dissolution of the bony trabeculae were present by the 6th day of the experiment, two days previous to the biological assay of parathyroid hormone-like activity in the sera. The parathyroid hypertrophy and the extensive bone dissolution, evident by the 10th and 15th days of the experiment, are shown in Figure 1.

Under the experimental conditions of the present study, calcium phosphate was precipitated in the kidney by the second day of the C-D diet (Fig. 1). The calcium deposition was progressive throughout the experiment resulting in considerable kidney damage by the 29th day of the experiment.

DISCUSSION

Parathyroid hormone-like activity was not assayable in the sera of rats fed the calcium -deficient, vitamin D-free diet until the eighth day of the experiment, although hypertrophy of the parathyroid glands was evident by the sixth day of the experiment.

Tetany was not observed in the rats maintained for 29 days on the calcium deficient, vitamin D-free diet. The maintenance of normal blood calcium levels was at the expense of bone salts, bone dissolution being evident by the sixth day of the experiment and progressing to such an extent in rats on the diet for 15 days that the trabeculae in the shafts of the tibias appeared as fragments. The only tetany observed was in thyroparathyroidectomized assay rats with a serum calcium in the 6.0 to 7.5 mg.% range when subjected to light ether anesthesia.

The lowest level of parathyroid hormone-like activity assayed was 10.5 units in a 0.5 cc. sample of serum from a rat on the diet for 10 days. The highest level of parathyroid hormone-like activity detected was 26.5 units in a 0.5 cc. serum sample from a rat on the diet for 29 days. A dose response was obtained when graded amounts of serum from rats on the diet were injected into the assay rats. The slope of the dose response curve was different than that obtained when standard parathyroid hormone was assayed. The assayable amount of parathyroid hormone-like activity, when expressed as

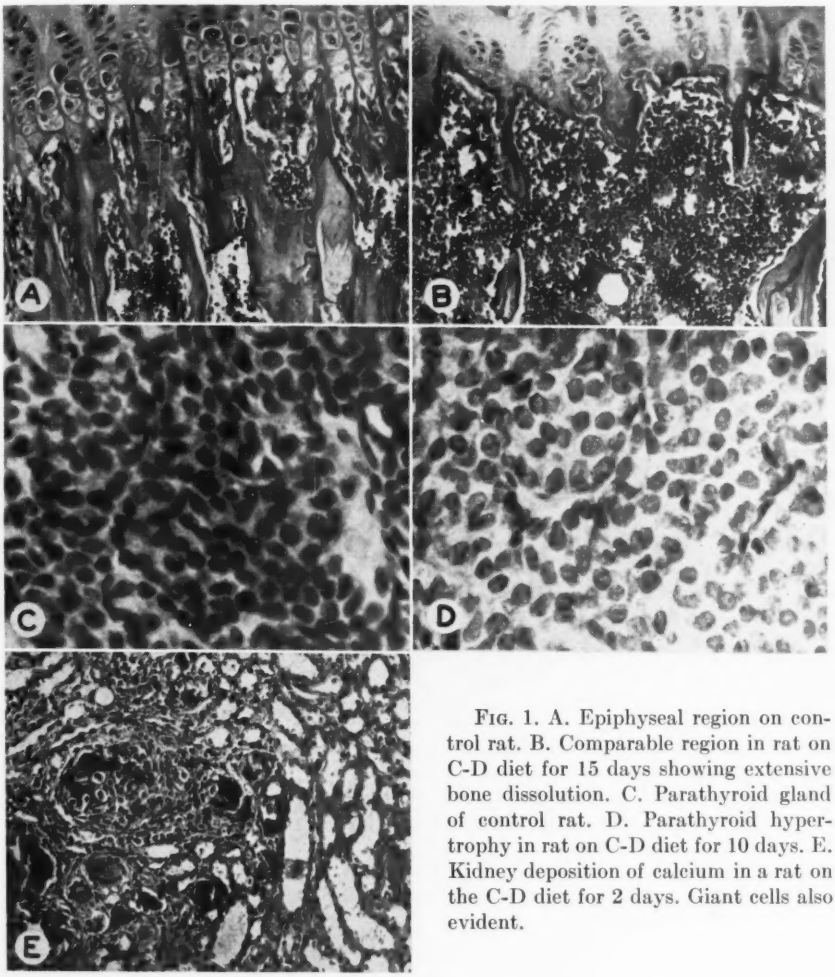

FIG. 1. A. Epiphyseal region on control rat. B. Comparable region in rat on C-D diet for 15 days showing extensive bone dissolution. C. Parathyroid gland of control rat. D. Parathyroid hypertrophy in rat on C-D diet for 10 days. E. Kidney deposition of calcium in a rat on the C-D diet for 2 days. Giant cells also evident.

units per cc. of serum assayed, decreased as the amount of serum injected into the assay animal increased. Rasmussen (19) reported that plasma or gelatin as an injection medium for parathyroid hormone causes a depression in the dose response curve and suggested that this may be due to a more rapid rate of absorption from the site of injection. In the present study, however, the serum-parathyroid hormone ratio remained constant as the amount of serum injected increased. The depression in activity is more likely due to inadequate absorption of parathyroid hormone from the subcutaneous depot.

Decreased levels of serum inorganic phosphorus are usually encountered in hyperparathyroidism, presumably due to the lowered renal threshold to

phosphate. Under the conditions in the present study, the high percentage of phosphate in the diet apparently created a heavy renal load and precipitation of calcium phosphate in the kidney tubules was evident by the second day of the experiment, even though the serum calcium was normal or slightly below normal. It is not surprising then that increased deposition of calcium phosphate can be correlated with increasing rise in serum inorganic phosphate levels. Urinary pH determinations revealed that the calcium-deficient, vitamin D-free diet produced a constant state of alkaline urine in the test rats, a condition conducive to calcium phosphate precipitation in the kidney. There was some concern subsequent to this study that the kidney damage encountered, and resulting uremia in the test rats, might influence the biological assay of the sera, that is, that uremic sera might influence the serum calcium levels in the parathyroidectomized assay rats and give a false positive parathyroid hormone response. In order to test this possibility, 1 cc. serum sample from rats bilaterally nephrectomized for two days, and containing nonprotein nitrogen values of 261 to 359 mg.%, were biologically assayed (20). There was no influence of sera from uremic rats on the serum calcium level in thyroparathyroidectomized assay rats.

This study demonstrated the quantitative detection of parathyroid hormone-like activity in the sera of rats with induced hyperparathyroidism. The biological assay of parathyroid hormone-like activity in the sera of the test rats was in all cases concomitant with hypertrophy of the parathyroid glands and evidence of peripheral parathyroid hormone activity, that is, associated with active bone dissolution.

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ROLE OF REDUCING AGENTS, ADRENAL GLANDS, AND PITUITARY GLANDS ON THE CONVERSION OF FOLIC ACID TO CITROVORUM FACTOR[1]

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ABSTRACT

The recovery of citrovorum factor present in the urine of male rats receiving folic acid is enhanced both by *in vitro* addition of ascorbic acid to the collection bottles and by *in vivo* stomach tube feeding of ascorbic acid. *In vitro* addition of glutathione or homocysteine produces the same increase in the recovery of citrovorum factor in the urine as that obtained with ascorbic acid while added cysteine is less effective.

Adrenalectomy lowers the urinary excretion of preformed citrovorum factor by male rats receiving folic acid but does not affect the bulk of the citrovorum factor activity present in urine in the form of a heat-labile compound (N^{10} formyltetrahydrofolic acid). Hypophysectomy lowers the urinary excretion both of preformed citrovorum factor and of the heat-labile precursor. Administration of beef-growth hormone to the hypophysectomized rats almost doubles the excretion of citrovorum factor in male rats receiving folic acid. However, administration of adrenocorticotropic or thyrotropic hormone to these rats does not affect the excretion of citrovorum factor.

THE administration of ascorbic acid to rats receiving folic acid has been reported by Welch *et al.* (1) to enhance the urinary excretion of citrovorum factor (CF). Dietary homocysteine or cysteine also increases the urinary excretion of CF[2] by rats receiving folic acid (2). Silverman and Gardiner (3) reported the occurrence in human urine of a factor (N^{10} formyltetrahydrofolic acid) which is labile to heat and alkali and which is converted to CF by autoclaving in the presence of ascorbic acid. A previous report (4) presented data which indicated that after administration of a test dose of folic acid to rats, about 20% of the increase in total CF activity excreted in the urine can be accounted for as CF, and the remain-

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[2] The abbreviation used is: CF, citrovorum factor.

ing 80% is present in the form of a heat-labile compound which upon autoclaving in the presence of ascorbic acid gives CF.

Both liver storage of folic acid and CF and the conversion of ingested folic acid to liver CF are reported (5) to be normal in thyroidectomized rats. Thyroid treatment, however, increases the liver folic acid (5) and impairs CF formation from folic acid. Adrenalectomy or hypophysectomy is reported (4) to lower the urinary excretion of CF by rats receiving folic acid. The present report is concerned with the role of reducing agents, the adrenal glands and the pituitary glands on the excretion of CF by rats receiving folic acid.

MATERIALS AND METHODS

Adult male rats of the Sprague-Dawley strain were placed individually in stainless steel metabolism cages equipped with specially designed collecting funnels (made by Micro-Metric Instrument Company). Rats were maintained on Purina laboratory chow throughout the experimental period. The animals were weighed and randomly distributed into groups of 6 each. Urine collections were started 24 hours after the rats had been placed on the above mentioned diet and were continued daily for 1 to 2-week test periods. Two drops of a 10% solution of thymol in isopropanol were added as a preservative to each collection bottle before beginning the daily urine collection. Addition of ascorbic acid to the urine collection bottles was optional and was included in the experiments where it was necessary to protect the heat-labile precursor in the urine during collection and to convert it to CF during the autoclaving procedure. For the determination of CF, the six 24-hour urine collections from 6 rats of the same group were combined and an aliquot of the pooled sample was filtered, neutralized, and autoclaved for 30 minutes at 120° C. The heated samples were cooled, reneutralized, diluted and assayed for CF content by using the Bacto-CF Assay Medium (Difco Laboratories, Inc.). *Pediococcus cerevisiae* ATCC 8081 was used as the test organism. Leucovorin (Lederle) was used as the standard. The cultures were incubated for 48 hours at 37° C. and growth was measured by a turbidimetric procedure. Since Leucovorin has been reported to be one-half as active as the CF isolated from horse liver (6), the microbiological assay values were divided by 2 in order to express the results in terms of the naturally occurring CF.

Hypophysectomized rats were obtained commercially (Hormone Assay Laboratories, Inc. Chicago, Illinois). Adrenalectomies were performed by the usual procedure. The adrenalectomized rats and the sham operated controls were given 1% salt in their drinking water.

RESULTS AND DISCUSSION

The results on the *in vitro* and *in vivo* effect of ascorbic acid on the recovery of CF in the urine of male rats receiving folic acid are shown in Figure 1. All the rats received 100 μg. of folic acid by stomach tube daily. Various levels of ascorbic acid were either put in the urine collection bottles or were administered by stomach tube daily. Urine samples from the 6 rats of each group were pooled and autoclaved before CF determinations were made. It is apparent from Figure 1 that only a small percentage of the administered folic acid is excreted in the urine as preformed CF. The bulk of the CF content is present as a heat-labile precursor. Addition of ascorbic acid to the urine collection bottles or administration of ascorbic acid by

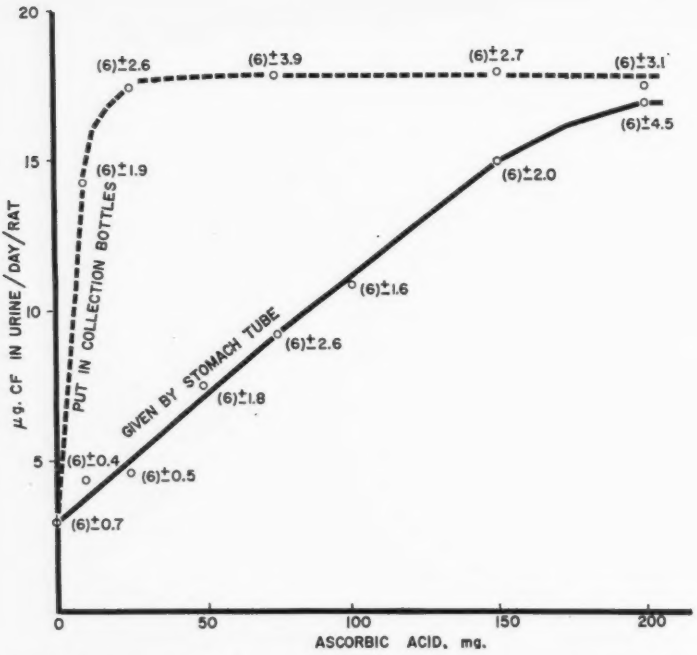

FIG. 1. Effect of ascorbic acid *in vitro* and *in vivo* on the recovery of CF in the urine of male rats receiving folic acid.

Rats were given 100 μg. folic acid by stomach tube daily and maintained on Purina Laboratory chow throughout the experiment. The figures by the side of each point on the graph represent the number of rats in each group. The urine samples were pooled and autoclaved prior to CF determinations. The values plotted are average daily CF recovery from a 7-day test period. Daily variations are shown by ± the standard deviation of the mean.

stomach tube enhances the recovery of CF in the urine presumably by protecting the heat-labile precursor during urine collection and by enhancing its conversion to CF during the autoclaving procedure. Figure 1 shows that 200 mg. ascorbic acid solution administered by stomach tube yielded the same recovery of CF in the urine as 25 mg. ascorbic acid solution placed inside the urine collection bottles. These results rule out the implication that dietary ascorbic acid enhances enzymatically the *in vivo* conversion of folic acid to urinary CF (1).

Studies on the *in vitro* effect of various reducing agents on the recovery of CF in the urine are presented in Table 1. Homocysteine and glutathione are as effective as ascorbic acid, whereas cysteine is less effective. Silverman and Gardinery (3) reported that autoclaving human urine samples with cysteine gave CF values lower than those obtained by autoclaving them with ascorbic acid. Dietary homocysteine is reported to be more

TABLE 1. EFFECT OF REDUCING AGENTS *in vitro* ON THE CONVERSION OF FOLIC ACID TO URINARY CF

Rats[1]			Addition to the collection bottles	Urinary excretion of CF,[2] μg. per day per rat
Group	No. per group	Aver. weight, gm.		
1	6	245	None	4.3 ± 1.3
2	6	254	Ascorbic acid, 150 mg.	21.6 ± 3.3
3	6	252	Homocysteine, 150 mg.	22.7 ± 1.5
4	6	259	Glutathione, 150 mg.	19.2 ± 1.6
5	6	257	Cysteine Hcl., 150 mg.	8.7 ± 1.2

[1] The rats were given 100 μg. folic acid by stomach tube daily and were maintained on Purina laboratory chow throughout the experiment.
[2] Figures for CF are mean daily excretion from a 7-day test period ± the standard deviation.

effective than cysteine in increasing the CF content of the urine from rats receiving folic acid (2).

Data concerning the effect of adrenalectomy on the urinary excretion of CF by male rats are presented in Table 2. During the first week of the experiment reported in Table 2, the urine samples were collected in empty bottles and later autoclaved at 120° C for 30 minutes prior to running the CF assay. This means that only preformed CF activity is determined. The results of this study (Table 2, first week) confirm the previous report (4) that adrenalectomy lowers both the excretion of CF and the *in vivo* conversion of folic acid to urinary CF. Also, cortisone acetate enhanced the excretion of CF in adrenalectomized rats receiving folic acid but similar treatment of sham operated animals was without effect (4). In order to determine the effect of adrenalectomy on the excretion of heat-labile precursor of CF and also on the conversion of folic acid to the presursor, during the second week of the experiment the urine samples were collected

TABLE 2. EFFECT OF ADRENALECTOMY ON URINARY EXCRETION OF CF BY MALE RATS

Rats			Supplement[1] to Purina chow per day per rat	Urinary excretion of CF,[2] μg. per day per rat	
Group	No. per group	Aver. weight, gm.		1st week	2nd week
1	6	324	Sham operation	0.4 ± 0.1	2.7 ± 0.4
2	6	323	+ Folic acid, 100 μg.	2.2 ± 0.2	21.0 ± 3.3
3	6	324	+ Folic acid, 100 μg. + Cortisone acetate, 2 mg.	2.1 ± 0.1	22.7 ± 1.2
4	6	304	Adrenalectomy	0.2 ± 0.1	1.8 ± 0.2
5	6	301	+ Folic acid, 100 μg.	1.2 ± 0.2	20.7 ± 2.2
6	6	309	+ Folic acid, 100 μg. + Cortisone acetate, 2 mg.	1.8 ± 0.3	21.5 ± 1.1

[1] Folic acid was administered by stomach tube, whereas cortisone acetate was given by intramuscular injection.
[2] The figures for CF are mean daily excretion from a 7-day test period ± standard deviation. During the 2nd week the rat urines were collected in bottles containing 300 mg. ascorbic acid.

TABLE 3. EFFECT OF GROWTH HORMONE ON THE CONVERSION OF FOLIC ACID TO URINARY CF BY HYPOPHYSECTOMIZED MALE RATS

Rats			Supplement[1] to Purina Chow per day per rat	Urinary excretion of CF,[2] μg. per day per rat		
Group	No. per group	Aver. weight, gm.		1st wk.	2nd wk.	Aver.
1	6	219	Sham operation	1.2±0.4	1.5±0.4	1.4
2	6	220	+Growth hormone, 0.5 mg.	1.4±0.2	1.9±0.5	1.6
3	6	223	+Folic acid, 100 μg.	12.6±0.4	14.6±1.7	13.6
4	6	215	+Folic acid, 100 μg.+ growth hormone, 0.5 mg.	11.5±2.0	13.7±1.9	12.6
5	6	171	Hypophysectomy	0.3±0.1	0.4±0.1	0.4
6	6	180	+Growth hormone, 0.5 mg.	0.4±0.1	0.5±0.1	0.4
7	6	175	+Folic acid, 100 μg.	4.6±0.8	4.3±0.6	4.4
8	6	181	+Folic acid, 100 μg.+ growth hormone, 0.5 mg.	6.7±0.9	8.3±0.8	7.5

[1] Folic acid was administered by stomach tube, growth hormone was given by intramuscular injection.

[2] Urine sample from each rat was collected in 300 mg. of ascorbic acid solution per bottle. Figures for CF are mean daily excretion from a 7-day test period ± standard deviation.

in 300 mg. of ascorbic acid solution, autoclaved at 120° for 30 minutes and assayed for CF. It is apparent from the results (Table 2) that adrenalectomy does not appreciably influence the excretion of the total CF[3] or the *in vivo* conversion of folic acid to its head-labile precursor. This indicates that the lowering of the *in vivo* conversion of folic acid to urinary preformed CF in adrenalectomized rats may be due to a lack of the adrenal glands.

The data on the effect of hypophysectomy on the urinary excretion of CF by male rats are reported in Table 3. Since urine samples from each rat were collected in 300 mg. of ascorbic acid solution per bottle, the values are for total urinary CF. Hypophysectomized rats excrete only one third as much CF as normal rats; similarly their conversion of folic acid to urinary CF is one third that of normals. Administration of growth hormone alone does not affect the excretion of CF by hypophysectomized or normal rats. However, when given together with folic acid, the growth hormone enhances almost 2-fold the conversion of folic acid to CF by hypophysectomized rats but is without effect in normals. The effect of growth hormone administration on the weights of the hypophysectomized rats are plotted in Figure 2. Normal animals subjected to sham operations gained on the average 58 gm. during the two-week period as contrasted to 42 gm. gained by hypophysectomized rats receiving 0.5 mg. growth hormone per day per rat. The weights of the hypophysectomized rats not receiving the growth hormone remained unchanged.

The effect of adrenocorticotropic hormone (ACTH), thyrotropic hormone (TSH) and extracts of the posterior lobe of the pituitary (Pituitrin-s)

[3] Total CF includes CF present in the urine and the CF formed from the heat-labile precursor by autoclaving the urine in presence of ascorbic acid.

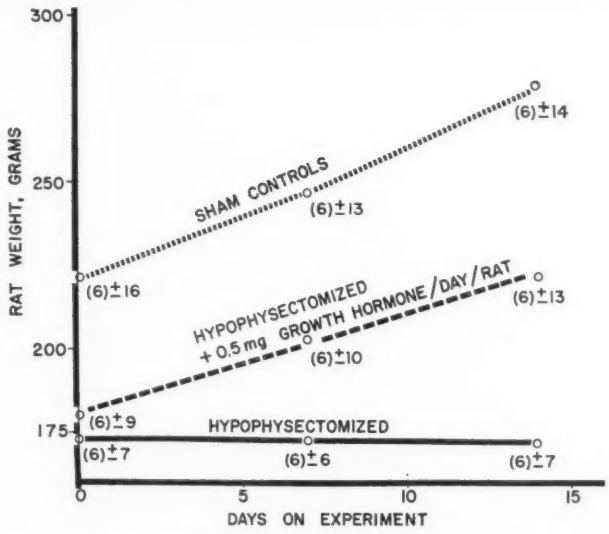

FIG. 2. Effect of growth hormone administration on the weights of the hypophysectomized rats during a two-week experiment. CF data for this experiment are described in Table 3. The figures by the side of each point on the graph represent the number of rats in each group. Values plotted are the average weights of the rats and the range is shown by ± the standard deviation of the mean.

TABLE 4. EFFECT OF ADRENOCORTICOTROPIC (ACTH), THYROTROPIC (TSH) AND EXTRACTS OF POSTERIOR LOBE OF THE PITUITARY (PITUITRIN-S) ON THE CONVERSION OF FOLIC ACID TO URINARY CF BY HYPOPHYSECTOMIZED MALE RATS

Rats			Supplement[1] to Purina Chow per day per rat	Urinary excretion of CF,[2] μg. per day per rat
Group	No. per group	Aver. weight, gm.		
			Hypophysectomized	
1	6	185	+Folic acid, 100 μg.	6.3 ±0.8
2	6	186	+Folic acid, 100 μg. +ACTH, 1 U.S.P. unit	6.1 ±0.4
3	6	186	+Folic acid, 100 μg. +TSH, 0.2 U.S.P. unit	6.6 ±0.9
4	6	173	+Folic acid, 100 μg. +Pituitrin (S), 2 U.S.P. unit	4.4 ±0.7
5	6	171	+Folic acid, 100 μg. +growth hormone, 0.5 mg.	11.4 ±0.8
6	6	173	+Folic acid, 100 μg. +growth hormone, 0.5 mg. +ACTH, 1 U.S.P. unit	10.7 ±1.4
7	6	185	+Folic acid, 100 μg. +growth hormone, 0.5 mg. +TSH, 0.2 U.S.P. unit	6.6 ±0.6

[1] Folic acid was administered by stomach tube, whereas ACTH, TSH, Pituitrin-S and growth hormone were given by intramuscular injections.

[2] Urine sample from each rat was collected in 300 mg. of ascorbic acid solution per bottle. The figures for CF are mean daily excretion from a 7-day test period ± standard deviation.

on the conversion of folic acid to urinary CF by hypophysectomized male rats are reported in Table 4. Administration of ACTH or TSH does not affect the conversion of folic acid to urinary CF, while growth hormone enhances it and Pituitrin-s inhibits it. The increase in the conversion produced by growth hormone is not affected by simultaneous administration of ACTH, but TSH appears to counteract the stimulatory effect of the growth hormone.

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RELATIONSHIP OF VITAMIN B_6 TO GLUCONEOGENIC ACTION OF CORTISOL[1]

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ABSTRACT

Recent investigations have shown that cortisol administration results in a marked increase in liver glutamic-pyruvic transaminase enzyme activity. Because of this observation it has been suggested that gluconeogenic action of adrenal steroids may depend upon elevation of this transaminase level. Since pyridoxal phosphate is the cofactor for the enzyme, effects of cortisol on liver glycogen deposition and glutamic-pyruvic transaminase activity were studied in vitamin B_6 deficient and control rats. The findings demonstrate that glucocorticoid action of cortisol is diminished in B_6 depleted animals as is the effect of the hormone on transaminase activity. Other parameters of cortisol action such as involution of lymphoid tissue and inhibition of ACTH release were not altered by the vitamin deficiency. It is concluded that gluconeogenic activity of adrenal corticoids depends not only on their molecular configuration but also upon nutritional status of the animal. The findings also support the concept that glucocorticoid activity may depend on increased levels of liver glutamic-pyruvic transaminase.

RECENT evidence has demonstrated that administration of cortisol or certain other adrenal steroids results in a marked increase in activity of the liver glutamic-pyruvic transaminase (LGPT) enzyme (1, 2). This effect appears to be specific since activity of a closely related enzyme, glutamic-oxalacetic transaminase, is altered only slightly in response to these hormones (3). Furthermore, only those adrenal steroids which have pronounced effects on carbohydrate metabolism are capable of stimulating LGPT activity (4). Because of the of these observations it has been suggested (2) that gluconeogenic action of adrenocortical hormones may depend upon increased activity of the LGPT enzyme system which results from their administration. If gluconeogenic action of adrenal steroids depends upon increased levels of LGPT, glucocorticoid activity of these hormones should be less in Vitamin B_6 deficient animals than in normal animals since pyridoxal phosphate is the cofactor for the enzyme. The experiments reported here were carried out to compare the effect of cortisol

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on liver glycogen deposition in B_6 deficient rats with that in intact animals and to determine if gluconeogenic activity of the steroid can be correlated with levels of glutamic-pyruvic transaminase. The results demonstrate that liver glycogen deposition following cortisol is less in B_6 deficient than in intact rats and that there is a direct correlation between amount of glycogen deposited and the rise in LGPT.

MATERIALS AND METHODS

Male rats (Holtzman) weighing 50–60 grams were fed a synthetic diet containing all known nutrients except pyridoxine. A control group was pair-fed the same diet to which pyridoxine (22 mg./kg.) was added. Animals were housed individually and weighed twice weekly. Both groups were maintained on their respective diets for 52 days. During the last week, half the animals of each group were given 5 mg. cortisol subcutaneously daily while those remaining were injected with the vehicle. Immediately before injection cortisol was suspended in the vehicle, a mixture of 0.5% carboxymethylcellulose, 0.4% Tween 80, and 1.5% benzyl alcohol dissolved in isotonic saline. On the final day, food was withheld from all animals and ten hours before they were sacrificed an additional injection of cortisol or vehicle was given. Animals were decapitated and spleen, adrenals, and liver removed and weighed. Spleen weight was used to determine the effect of cortisol on lymphoid tissue and adrenal weight served as a measure of the pituitary inhibiting effect of the hormone. Duplicate samples of liver weighing approximately 200 mg. were taken and glycogen content determined by the method of Walaas and Walaas (5). The remainder of the liver was homogenized with 19 volumes of cold distilled water and stored at −20° C. Subsequently, LGPT activity of the homogenate was measured using a procedure described by Lowry (6). Protein content of each liver was also determined (7).

RESULTS

Animals which consumed the B_6 deficient diet gained weight throughout the experiment, although the rate of gain was slower than that of the pair-fed controls (Table 1). During the last week when cortisol was administered, rats of both groups which received the hormone lost weight. Weight loss was greater in deficient animals than in controls. Administration of cortisol to pyridoxine-depleted as well as control animals resulted in involution of the spleen and atrophy of the adrenals (Table 1). In both groups, spleen weight of animals injected with cortisol was less than half

TABLE 1. EFFECT OF CORTISOL ON BODY, SPLEEN AND ADRENAL WEIGHTS OF B_6 DEFICIENT AND PAIR-FED CONTROL RATS

	Final body weight (gm.)	Spleen weight (mg.)	Adrenal weight (mg.)
Pair-Fed Control Rats Injected with Vehicle (16)	177.5 ± 2.5	346.1 ± 7.6	37.56 ± .87
Pair-Fed Control Rats Injected with Cortisol (16)	156.4 ± 1.4	169.7 ± 5.5	20.49 ± .73
B_6 Deficient Rats Injected with Vehicle (13)	146.8 ± 5.6	308.5 ± 11.1	35.37 ± 1.44
B_6 Deficient Rats Injected with Cortisol (15)	113.4 ± 3.5	115.3 ± 5.7	21.03 ± .75

Mean values ± standard error of mean.
Number of animals in group in parenthesis.

that of those receiving the vehicle. Reduction of adrenal weight in rats receiving cortisol was of approximately the same magnitude in deficient and control groups. It should be pointed out, however, that if adrenal weight is considered in relation to body weight, the decrease is greater in control than in deficient rats. This can probably be explained by the greater degree of body weight loss in the B_6 deficient group.

Cortisol administration to pair-fed control rats resulted in a 9-fold rise in hepatic glycogen concentration and total liver glycogen was increased almost 20 times (Table 2). When the hormone was given to B_6 deficient rats, however, increases in liver glycogen concentration and total glycogen content were considerably smaller. Differences in glycogen deposition following corticol between the two groups were significant, $P = <0.001$.

TABLE 2. EFFECT OF CORTISOL ON LIVER WEIGHT, GLYCOGEN CONTENT, AND GLUTAMIC-PYRUVIC TRANSAMINASE ACTIVITY OF B_6 DEFICIENT AND PAIR-FED RATS

	Liver weight (gm.)	Liver glycogen (mg./gm.)	Total liver glycogen (mg./liver)	LGPT activity (mM DPN formed/total liver/hour)
Pair-Fed Control Rats Injected with Vehicle (16)	4.7 ± .08	8.87 ± .84	41.72 ± 4.28	33.3 ± 1.05
Pair-Fed Control Rats Injected with Cortisol (16)	9.2 ± .17	80.64 ± 2.18	738.9 ± 24.3	131.7 ± 3.27
B_6 Deficient Rats Injected with Vehicle (13)	4.7 ± .16	11.52 ± 1.34	54.43 ± 6.96	12.0 ± .74
B_6 Deficient Rats Injected with Cortisol (15)	4.9 ± .25	38.25 ± 6.59	205.9 ± 38.5	26.5 ± 1.38

Mean values ± standard error of the mean.
Number of animals in group in parenthesis.

LGPT level of deficient rats injected with the vehicle was much less than that of comparable controls and is indicative of the degree of vitamin B_6 depletion in these animals (Table 2). Cortisol injection in controls induced a marked increase in LGPT activity, the level rising from 33 mM DPN[2] formed/liver/hour to 132, whereas, in pyridoxine-depleted rats LGPT activity rose only from 12 to 26. This difference was statistically significant, $P = <0.001$.

Liver hypertrophy was observed in control rats receiving cortisol but did not occur in the deficient group (Table 2). Hepatic enlargement was due, in part, to increased glycogen levels but protein and water contents were also found to be elevated. Analysis of lipid content was not carried out.

DISCUSSION

The results of this investigation have demonstrated that when cortisol is administered to vitamin B_6 deficient rats, only small increases in liver glycogen and LGPT activity occur. The decreased glucocorticoid activity of

[2] Diphosphopyridine nucleotide.

cortisol can probably be attributed to diminished tissue content of pyridoxal phosphate which prevents an increase of transaminase activity in response to the hormone. It is postulated that since LGPT activity remains low, conversion of alanine to pyruvic acid is limited and, as a result, only small amounts of glycogen are synthesized. In control animals, cortisol administration resulted in a marked increase in LGPT activity and deposition of large quantities of glycogen in the liver. Thus, it is apparent that there was a direct correlation between the rise in transaminase activity and glycogen synthesis both in B_6 deficient and control rats. These results are in harmony with the suggestion that gluconeogenic action of adrenal steroids may be mediated through an increase in activity of the LGPT.

It is of interest that vitamin B_6 is concerned with glycogen metabolism not only through its role as coenzyme for LGPT but pyridoxal phosphate is also a cofactor for phosphorylase, an enzyme concerned primarily with glycogenolysis (8). Furthermore, previous evidence demonstrating the influence of vitamin B_6 on hormonal action has been obtained by Wooten *et al.* (9) who showed that metabolic effects of follicle-stimulating and interstitial cell-stimulating hormones were greatly reduced in pyridoxine-depleted rats. Even though the effects of cortisol, follicle-stimulating hormone and interstitial cell-stimulating hormone are inhibited by B_6 deficiency, there is no evidence to suggest that metabolic actions of all these hormones are mediated through the same B_6 dependent enzyme.

Involution of lymphoid tissue and inhibition of ACTH release, as determined by spleen and adrenal weights, were almost the same in the two groups although glycogen deposition and liver hypertrophy were greater in control than in deficient animals. These findings make it evident that certain metabolic effects of adrenal steroids can be altered while other actions remain unchanged. Increased liver weight after adrenocortical hormone administration has been observed by other investigators (10) but it has not been reported that the effect is abolished in pyridoxine deficiency. This finding cannot be explained by the results of the investigation reported here but it serves to emphasize that certain responses to hormones may be impaired by nutritional deficiency. The observation that certain actions of cortisol were altered while others remained intact suggests that cortisol may exert its various effects by influencing several enzyme systems. As a matter of fact, it has been shown that the hormone affects activity of several enzymes including D-amino acid oxidase, xanthine oxidase, glucose 6-phosphatase and others (3) although the significance of these changes is not understood.

Acknowledgment

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BIOASSAY OF RELAXIN USING A REFERENCE STANDARD: A SIMPLE AND RELIABLE METHOD UTILIZING DIRECT MEASUREMENT OF INTERPUBIC LIGAMENT FORMATION IN MICE

B. G. STEINETZ, V. L. BEACH, R. L. KROC, N. R. STASILLI, R. E. NUSSBAUM, P. J. NEMITH AND R. K. DUN[1]

ABSTRACT

The estrogen-primed mouse responds to single injections of relaxin with dose-proportional increases in the length of the interpubic ligament. This effect is specific for relaxin and has served as the basis for published bioassay procedures. A simple and accurate method for direct measurement of interpubic ligament length was previously developed, employing transillumination and magnification of the exposed pubic symphysis (13). A quantitative bioassay procedure was devised in which relaxin-containing preparations were evaluated in terms of a reference standard (13). The present report is an extension of this earlier work. Detailed evidence is presented to justify each step of the assay procedure. The data demonstrate that precision and reproducibility of the assay depends primarily on control of the following variables: selection of mice, adequacy of estrogen priming, selection of injection vehicle, rate of ligament formation, and seasonal shifts in responsiveness of mice. Thus, mice which fail to gain weight during the estrogen priming period are not reliable for assay. Uterine enlargement is correlated with the effectiveness of the estrogen priming, and is used as a criterion of assay eligibility. Rate of interpubic ligament formation is dependent on the vehicle used for relaxin injection. Several commercial strains of mice were used for assay weekly over a period of 2 years. Significant seasonal shifts in response to the relaxin reference standard were observed. Thus, unknowns must be evaluated in terms of a concomitant standard. Response units are unreliable.

When these precautions are used, limits of error of potency estimates generally fall within -27 to $+35\%$ at $p = 0.95$, and λ is 0.2–0.4.

THE protein-like ovarian hormone, relaxin, has not yet been isolated as a chemically identifiable substance. Consequently, demonstration of relaxin activity in animal tissues, and determination of potency of relaxin-containing extracts, have depended entirely upon biological assay methods.

Since the discovery of relaxin by Hisaw (1) in 1926, many bioassay methods have been proposed. These methods employ such diverse biological responses as "relaxation" of the pubic symphysis of guinea pigs (2, 3, 4) or mice (5, 6, 7, 8), and inhibition of uterine motility *in vivo* or *in vitro* in guinea pigs (9), rats (10) and mice (11, 12, 13). However, no one assay pro-

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[1] Deceased.

cedure has received universal acceptance. As a consequence, reports of basic experimental and clinical work on relaxin have been confused by a complete lack of standardization between preparations used by different investigators (14).

The major problems of relaxin bioassay have been discussed by many authors (7, 8, 13, 14, 15, 16). Basically, the difficulty is twofold: 1) There exists no widely accepted and available relaxin reference standard; 2) The classical method of assay (guinea pig pubic symphysis palpation) is difficult to reproduce, subjective, costly, and rigid statistical criteria have seldom been applied.

Elongation of the interpubic ligaments of mice has served as a satisfactory parameter of relaxin activity in the hands of many investigators. Indeed, assay procedures involving x-ray of the mouse pubic symphysis before and after treatment with relaxin have been proposed by Hall (5), Dorfman, *et al.* (6) and Kliman and co-workers (7, 8). Kliman and Greep (17) demonstrated the importance of a repository vehicle for relaxin injection in mice.

Kroc *et al.* (13) have reported briefly on a simplified mouse assay, in which time-consuming x-ray techniques are replaced by rapid and precise direct measurement of transilluminated pubic ligaments. Potencies were estimated in terms of a concomitant reference standard.

The present report is an extension of the work of Kroc *et al.* (13) and presents evidence justifying each step of the assay procedure and the use of a relaxin reference standard.

MATERIALS AND METHODS

A. *Animals.* Swiss albino virgin female mice, weighing 18–20 gm. are used for bioassay. Gentle, rapidly growing mice are superior to slow weight-gainers and inherently nervous strains. Several commercially available strains have been found satisfactory for assay purposes.[2] The mice are maintained uncrowded in groups of ten per box, fed Rockland Mouse Diet supplemented with whole oats twice per week, and allowed water ad libitum.

B. *Injections.* Estradiol cyclopentylpropionate[3] is diluted with peanut oil and injected subcutaneously in a dose of 5 μg./0.1 ml. to prime mice for assay use.

Relaxin extracts are routinely administered subcutaneously in 0.2 ml. of 1% L-390 (benzopurpurine 4B), an azo dye repository agent. Other vehicles studied include 0.9% saline, 5% beeswax in peanut or sesame oil, 0.5% Evans Blue Dye, 20% polyvinylpyrrolidone (PVP), 20% gelatin and 4% polyphloretin phosphate.

C. *Equipment.* A binocular dissecting microscope[4] is outfitted with a calibrated ocular micrometer and transilluminator (13). The transilluminator consists of a "U-shaped" lucite rod which protrudes through the stage to present its beveled tip directly beneath the objective. The light source used is a standard Nicholas illuminator.

[2] A. J. Barckmann & Son, Barnegat, N. J., Taconic Farms, Germantown, N. Y., Manor Farms, Staatsburg, N. Y., Green Hills Farms, Jeffersonville, N. Y., Flanders Farms, Flanders, N. J.

[3] Depo® Estradiol, Upjohn.

[4] Bausch and Lomb Model BKT-5 Stereomicroscope.

D. *General Procedure.* All mice receive a single subcutaneous injection of 5 μg. of estradiol cyclopentylpropionate in 0.1 ml. of peanut oil on day 0 (7 days prior to the desired assay day).

Relaxin preparations are injected subcutaneously on day 7. Eighteen to twenty-four hours later, mice are killed by exposure to an atmosphere of CO_2. The mice are weighed and the abdominal cavity is opened. Mice weighing less than 19 gm. or exhibiting thread-like uteri (failure of estrogen priming) are discarded. The vulval and anal areas are cut away, and the upper half of the body is cut off to prevent bleeding into the pelvic area. The bony birth canal is freed of skin, vagina and rectum, and the symphysis pubis is exposed for measurement.

The pelvis is positioned on the transilluminator so that the tip of the lucite rod passes a beam of light vertically through the exposed pubic ligament, as illustrated in Figure 1. The feet are grasped between the thumbs and index fingers, and a slight lateral traction is applied. (The ligaments do not stretch, but may tear if too much traction is used.) A micrometer reading is made of the shortest distance between the edges of the pubes. Measurements are accurate to ± 0.0375 mm. Micrometer readings are converted to mm. An experienced operator can dissect and measure at a rate of approximately 3 mice per minute.

E. *Relaxin Reference Standard.* In 1955, a 130 gm. lot of purified relaxin was set aside as a reference standard. This material asaayed 150 guinea pig units/mg. by old criteria of the guinea pig pubic symphysis palpation method (2, 13). The standard was designated Warner-Chilcott Relaxin Reference Standard W1164-A, Lot 8. This standard has also been referred to by its old laboratory number, B-37-348/8a1α (18, 19).

F. *Routine Assay Procedure.* A six point balanced assay design is generally employed, using twenty estrogen-primed mice for each of 3 dilutions of S (relaxin reference standard) and U (unknown). In practice, it has become advantageous to run 2 or more unknowns concomitantly with the standard. Thus, 180 to 240 mice are usually primed for each assay day.

1. Single s.c. injections of S and U are administered in 0.2 ml. of a 1% L-390 (Benzopurpurine 4B) solution.

2. Dose levels are two-fold apart. U doses are adjusted to approximate responses to S, after preliminary assay.

3. Responses are estimated from the mean pubic ligament length for each group and plotted against dose administered, on a semilogarithmic scale.

4. Statistical analyses are performed according to the methods of Bliss (20) and Snedecor (21). The calculated potency of U is expressed in terms of mg. equivalents to S. Limits of error of the potency estimate should fall within minus 33% and plus 50% at the 95% confidence level for an acceptable assay.

RESULTS

Repository vehicle. Approximately 50 compounds and formulations were tested for repository effectiveness with relaxin. Dose-response curves obtained with a few of the active agents are shown in Figure 2. Physiological saline proved to be an inefficient vehicle for relaxin injection. A 100–300-fold increase in dose was required to produce a strong response when the hormone was administered in saline rather than in beeswax-oil or in L-390.

1% L-390 and 5% beeswax in peanut oil have been adopted as the most useful vehicles in terms of steepness of slope and reduction of relaxin dose.

The rate of interpubic ligament elongation was estimated by sacrificing

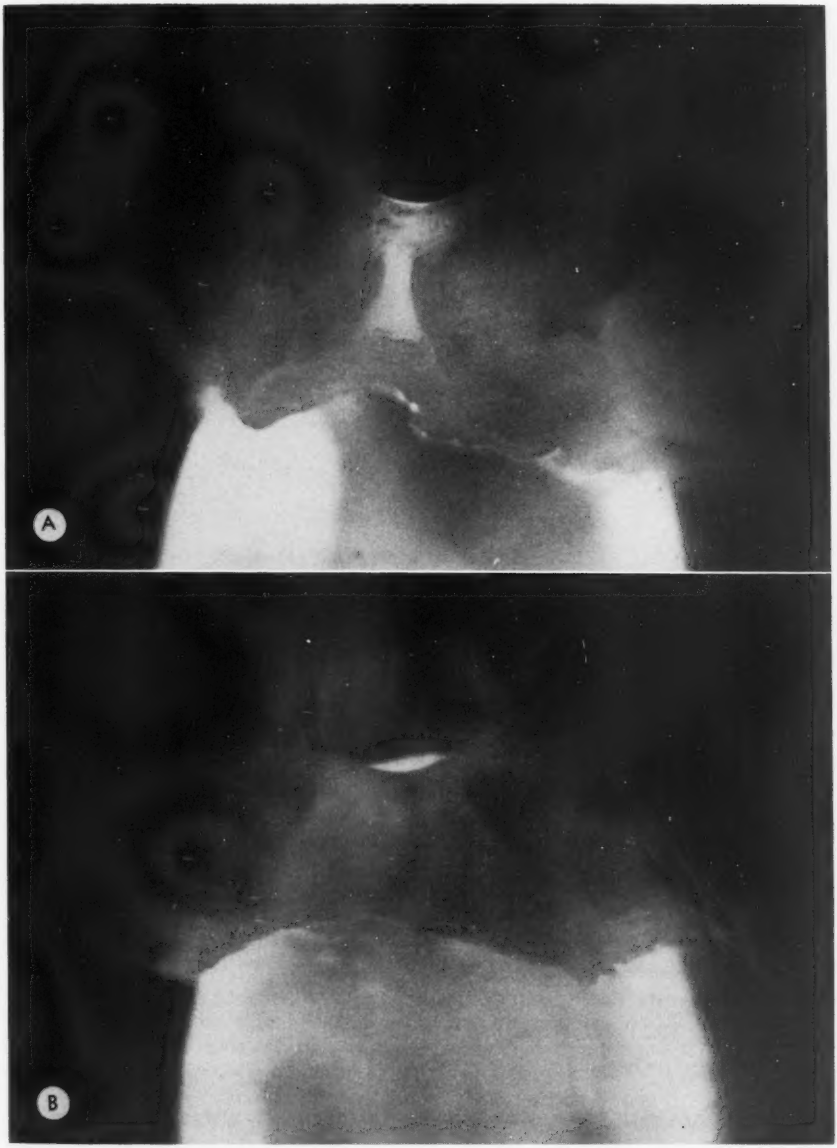

FIG. 1. Transilluminated mouse pubic symphysis as viewed through binocular dissecting scope at 13× magnification. (A) Mouse primed with 5 μg. estradiol cyclopentylpropionate 8 days previously. Ligament length was 0.30 mm. (B) Mouse primed with 5 μg. estradiol cyclopentypropionate 8 days previously, and treated with 3.3 μg. relaxin standard in 1% L-390 on day 7. Ligament length was 1.72 mm.

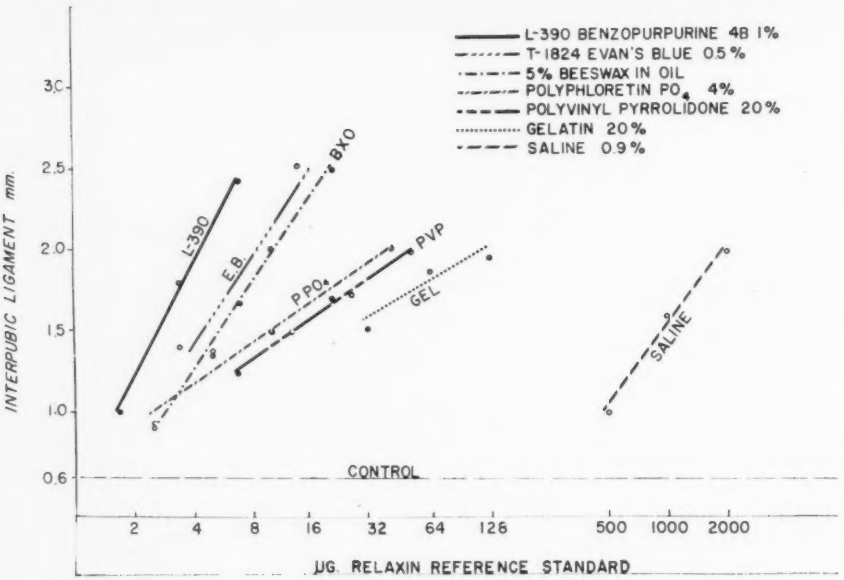

FIG. 2. Composite graph showing influence of vehicle on mouse pubic symphysis response to relaxin. Each point represents average ligament lengths of 10–20 mice. All mice primed with 5 µg. estradiol cyclopentylpropionate on day 0. Relaxin injected day 7. Vehicles alone did not influence ligament lengths, and control line was drawn from average of all controls (5–10 mice per vehicle).

groups of mice at various time intervals following a single injection of relaxin in saline solution or 1% L-390 (Fig. 3). The doses selected for each vehicle were approximately equivalent in terms of 24-hour total response. Ligament formation occurred at a rather uniform rate after injection of relaxin in saline. When the hormone was administered in L-390, only slight elongation was observed to occur prior to the 12th hour after injection. This was succeeded by extremely rapid lengthening during the next three hours. With either vehicle, ligament formation appeared to be complete by the 18th hour. (Fully-formed ligaments have not been observed to regress for periods up to 192 hours after injection (13).)

Factors affecting the absolute response of the mouse interpubic ligament

Colony sensitivity. The sensitivity of the mouse pubic symphysis response to relaxin may vary seasonally (Fig. 4). The cause(s) of reduced responsiveness has not been identified. However, the yearly pattern demonstrated the fallacy of relying on a response unit. Thus, in terms of absolute "mouse units," the relaxin reference standard "lost" 30–40% of its activity during the period January–March 1957, "gained" activity through December 1957, then "lost" or "gained" significantly depending upon strain used (Fig. 4).

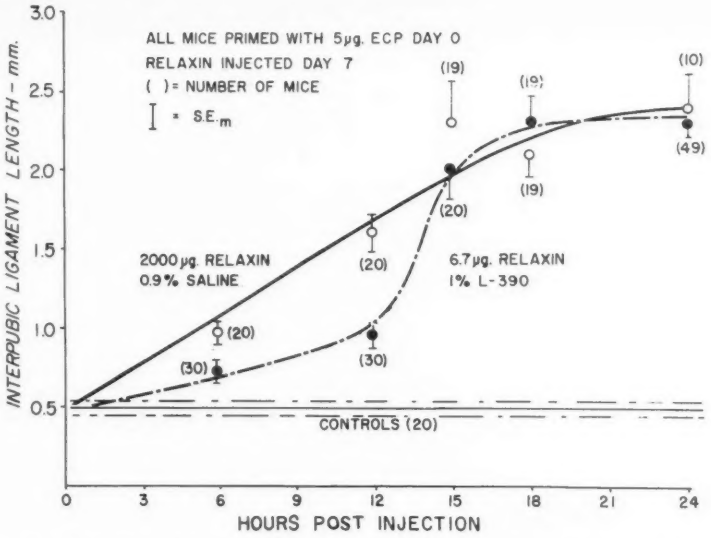

FIG. 3. Elongation of interpubic ligament following a single injection of relaxin in 0.85% saline or 1% L-390. All mice primed with 5 μg. estradiol cyclopentylpropionate day 0. Relaxin was injected day 7, and groups of mice were sacrificed for interpubic ligament measurement at the time intervals indicated. 10 controls were injected with 0.2 ml. saline and 10 controls received 0.2 ml. 1% L-390. Control lines represent average and standard error of the two groups.

Body weight. Gain in body weight between the time of estrogen priming and sacrifice may also influence absolute response to a given dose. In a typical experiment, mice were divided into different weight groups at the time of relaxin injection. Only $\frac{2}{3}$ of the mice weighing less than 22 gm. responded to the hormone, thereby reducing the mean ligament length for the group (Table 1). In the usual assay group, mice weigh 22–26 gm.; stunted animals are always discarded.

Extrogenization.[5] Inadequate estrogenization will reduce responsiveness

[5] Despite the small cyclical variations in interpubic ligament lengths of adult female mice noted by Kliman and Salhanick (32), the unprimed mouse does not respond to low doses of relaxin (see Table 2). Thus, the estrogen required for pubic symphysis responses to *low doses* of relaxin is apparently far in excess of normal production of the mouse ovary. In fact, the presence or absence of steroid-producing organs is irrelevant to the ligament response to small doses of relaxin (e.g., 6.7 μg.) in mice primed with 5 μg. ECP:

Operation	*Treatment*	*N*	*Ligament length, mm. ± S.E.*
None	Relaxin, 6.7 μg.	20	2.39 ± 0.22
Ovariex	Relaxin, 6.7 μg.	11	2.14 ± 0.08
Adrenalex (salt maintained)	Relaxin, 6.7 μg.	10	2.45 ± 0.22
None	None	10	0.55 ± 0.08

When no exogenous estrogen is administered, massive doses of relaxin (e.g., 1–5 mg.) are required to induce ligament formation.

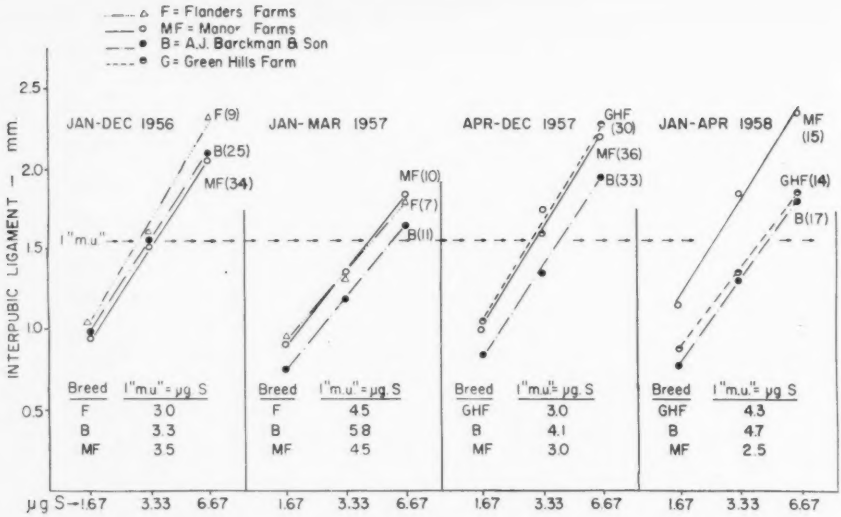

FIG. 4. Seasonal variations in responsiveness of mice to Warner-Chilcott relaxin standard ("S") W1164A, Lot 8. Numbers in parenthesis refer to number of assays. As approximately 60 mice were employed for each assay, the total number of mice used for establishing a single curve may easily be calculated by multiplying the number in parenthesis by 60 (e.g., GHF, April–Dec., 1957, $30 \times 60 = 1{,}800$ mice). Direct statistical analysis was not attempted because of the formidable numbers of animals. However, a reasonably accurate statistical prediction was made on the basis of routine assay analysis. Limits of error at $p = 0.95$ would be $\pm 15\%$ using 308 mice, $\pm 10\%$ with 686 mice and $\pm 5\%$ with 2,745 mice. For comparison of any two curves, one can thus estimate the probable combined assay error by totaling the number of assays and multiplying by 60 to obtain N. The limits of error may then be estimated from the predictions shown above. "m.u." refers to a hypothetical mouse unit, or the dose of relaxin necessary to produce a ligament length of 1.55 mm. (midpoint of curve, January 1956).

of mice to relaxin. Groups of mice were primed with graded doses of ECP in peanut oil, or peanut oil alone. Seven days later, all animals received 6.7 μg. of relaxin in 1% L-390. Mice were sacrificed 24 hours later, and interpubic ligament lengths and uterine weights were recorded. 5 μg. of ECP was observed to be the optimal priming dose (Table 2). Uterine weight increase paralleled interpubic ligament length, thus offering another criterion

TABLE 1. INFLUENCE OF BODY WEIGHT ON RESPONSE TO 6.7 μG. RELAXIN IN ESTROGEN PRIMED MICE

Body wt. group	No. of mice	Interpubic ligament length, mm. ±S.E.	% non-responders
<22 gm.	19	1.48 ±0.37	32%
22 gm.	35	2.03 ±0.15	3%
23 gm.	36	2.36 ±0.13	3%
>23 gm.	21	2.51 ±0.12	0%

TABLE 2. EFFECT OF ESTROGEN PRIMING ON MOUSE PUBIC SYMPHYSIS RESPONSE TO 6.7 μG. RELAXIN REFERENCE STANDARD IN 0.2 ML. 1% L-390*

Dose of estradiol cyclopentylpropionate, μg./0.2 ml. peanut oil	No. of mice	Uterine wt. mg. ±S.E.	Interpubic ligament mm. ±S.E.
0	18	40 ± 4	0.29 ± 0.03
1.25	18	127 ± 9	1.49 ± 0.17
2.50	20	142 ± 6	2.15 ± 0.20
5.00	19	182 ± 10	2.63 ± 0.16
10.00	18	178 ± 6	2.12 ± 0.20

* Estrogen injected day 0, relaxin injected day 7.

for assay eligibility. Therefore, mice which exhibit threadlike uteri (an indication of priming failure) at autopsy are always discarded without exposing the pubic symphysis. While it has not proved necessary to run estrogen controls (primed mice receiving only vehicle) with every assay, it is important to make periodic checks on control ligament lengths. The lowest dose of relaxin used should induce approximately a 2-fold increase in ligament length over estrogen controls (Table 3).

Strain. Many commercial strains of mice have been used successfully for relaxin bioassay. The yearly variations in these animals (Fig. 4) make it unwise to attribute to heredity bizarre results occasionally obtained with sample mice from other suppliers.

Specificity. Only relaxin-containing preparations induced interpubic ligament formation in mice under the conditions described. Control tests

TABLE 3. COMPARISON OF CONTROL AND RELAXIN-INFLUENCED LIGAMENT LENGTHS IN ESTROGEN PRIMED MICE

	Relaxin dose, μg., in 0.2 ml. 1% L-390			
	0	1.67	3.3	6.7
Ligament length, mm., ±S.E.	0.48 ± 0.10 (6)	1.01 ± 0.13 (20)	1.81 ± 0.19 (20)	2.43 ± 0.20 (18)
	0.67 ± 0.25 (9)	0.98 ± 0.08 (20)	1.35 ± 0.12 (20)	2.06 ± 0.13 (20)
	0.74 ± 0.18 (9)	1.50 ± 0.18 (10)	1.90 ± 0.24 (20)	2.40 ± 0.17 (20)
	0.45 ± 0.14 (10)	0.99 ± 0.24 (20)	1.79 ± 0.19 (20)	2.10 ± 0.13 (20)
	0.55 ± 0.07 (10)	0.96 ± 0.08 (19)	1.42 ± 0.10 (19)	2.05 ± 0.18 (19)
	0.59 ± 0.10 (10)	0.98 ± 0.07 (20)	1.63 ± 0.12 (19)	2.06 ± 0.05 (19)
	0.50 ± 0.06 (10)	1.09 ± 0.08 (20)	1.58 ± 0.12 (20)	2.07 ± 0.12 (20)
	0.62 ± 0.14 (9)	1.22 ± 0.19 (10)	1.80 ± 0.23 (16)	2.28 ± 0.23 (17)

TABLE 4. SPECIFICITY OF 3 METHODS OF RELAXIN BIOASSAY RESPONSES (0 = NO EFFECT; + = POSITIVE; − = INHIBITS RELAXIN EFFECT)

Preparation	Interpubic ligament formation in mice	Relaxation of guinea pig pubic symphysis	Inhibition of estrous mouse uterine motility *in vitro*	Remarks
Relaxin	+	+	+	Sources of relaxin—pregnant rabbit serum, swine ovaries
17β-estradiol	0 (±)*	0 (+)[22]	0	17β-estradiol induces bone resorption and/or ligament effects upon prolonged administration. No effect in usual test interval for guinea pigs and mice
Progesterone	0 (−)[23,24]	0 (+)[22]	+	Progesterone induced endogenous formation of relaxin in guinea pigs. No effect in usual test interval
DCA	0 (−)[24]	0 (+)[25]	Not tested	Similar to progesterone
Cortisone	0 (−)[24]	Not tested	Not tested	
Elastase	0	0	+ (atypical)	
Gonadotrophins (pituitary or chorionic)	0 (−)[24]	0 (+)[26]	0	Gonadotrophins relaxed guinea pig symphysis pubis after prolonged administration. No effect in usual test interval
Growth Hormone	0	Not tested	Not tested	
ACTH	0 (−)[24]	0[27]	Not tested	
Thyroid Hormone	0 (+)[28]	Not tested	0	Pretreatment with desiccated thyroid enhanced mouse pubic symphysis response to injected relaxin
Relaxin-type extract of boar testis	0	0	0	

* Parentheses indicates possible result under experimental conditions different from those used for assay. Unless indicated by reference, data were obtained by the authors and is unpublished.

and comparison with the specificity of the guinea pig symphysis pubis and uterine motility methods are summarized in Table 4.

Typical assay results. Figure 5 illustrates dose-response curves and statistical evaluation of a typical mouse assay. The relaxin reference standard W1164A, Lot 8 (S), and unknown Lot 14426 (U), were injected in 0.2 ml. of 1% L-390. Lot 14426 had been sterilized by filtration and vialed at a theoretical activity of 20 mg.S/ml. based on previous bioassay of its constituent powder. The observed potency of Lot 14426 was 22.8 mg.S/ml.

Table 5 illustrates the bioassay of W1164-34, Lot 0729, a vialed, sterilized repository suspension. This assay represents the maximum precision

TABLE 5. MOUSE PUBIC LIGAMENT BIOASSAY OF RELEASIN RESPIRATORY SUSPENSION W1164-35, LOT 0729 (U) VS. RELAXIN REFERENCE STANDARD W1164-A, LOT 8 (S)

Preparation	No. of mice	Dose, mg. or ml./mouse	Av. ligament length, mm. ± S.E.
S	20	1.67 mg.	1.25 ± 0.12
S	19	3.33 mg.	1.88 ± 0.11
S	20	6.67 mg.	2.81 ± 0.13
U	21	1/12,000 ml.	1.16 ± 0.10
U	21	1/ 6,000 ml.	1.73 ± 0.12
U	19	1/ 3,000 ml.	3.14 ± 0.13

Mean observed potency of U: 20.4 mg.S/ml.
Range of potency (at P = 0.95): 17.5–23.7 mg.S/ml.
Limits of error (at P = 0.95): 86–116%
S = 0.527 b = 2.95 λ = 0.179

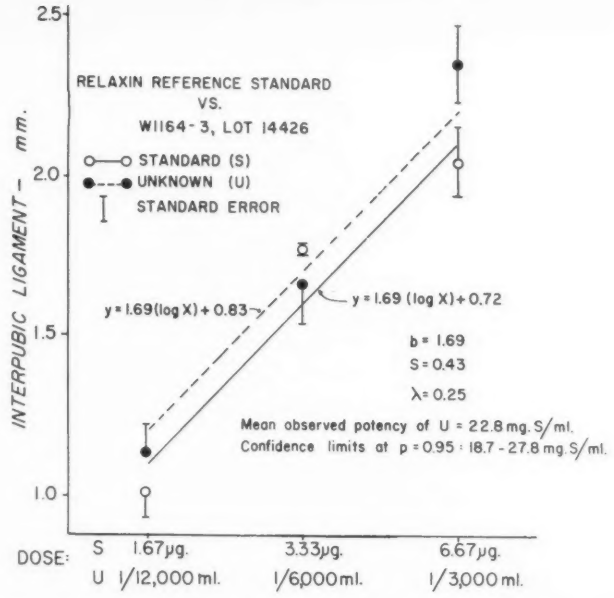

FIG. 5. Bioassay of sterile-vialed relaxin (Releasin®) solution W-1164-3, Lot 14426. 120 mice were equally divided among 3 dose levels each of Standard and Unknown. b = slope, s = standard error of the assay, λ = index of precision (s/b).

thus far obtained with the mouse method. Table 6 lists results, reproducibility and statistical evaluations of 9 mouse assays performed in duplicate. The greatest discrepancy between potency estimates of duplicate assays was 16% (not significant). The average replicate value for the 9 assays was 91%.

Figure 6 illustrates the distribution of lambda values obtained in 21 complete assays of powders and vialed solutions. Lambda was observed to be 0.3 or less in $\frac{2}{3}$ of the assays.

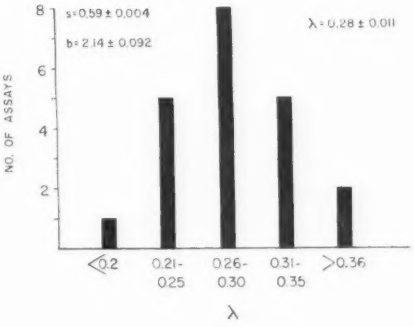

FIG. 6. Distribution of lambda values in 21 mouse assays. In two-thirds of the assays, lambda was 0.30 or less, s and b are defined in Figure 5.

TABLE 6. RESULTS OF DUPLICATE MOUSE ASSAYS OF RELAXIN-CONTAINING EXTRACTS AGAINST REFERENCE STANDARD W1164-A, LOT 8

Type of preparation	Total mice	Potency of U, mg. S/ml. or mg.	Limits of error, % at P = 0.95	λ
Sterile Vialed Solutions:				
Lot 22907 a)	113	24.2	79–127	0.28
b)	112	20.6	81–124	0.24
Lot 25367 a)	120	23.8	82–123	0.24
b)	120	26.0	76–131	0.32
Lot 24757 a)	119	21.2	71–141	0.41
b)	120	19.3	80–125	0.27
W1164–35 Lot 0729 a)	118	19.3	84–119	0.21
b)	120	20.4	86–116	0.18
Powders:				
M-9714 a)	121	1.43	74–135	0.36
b)	117	1.51	78–129	0.31
M-8630 a)	117	0.88	80–124	0.25
b)	115	0.74	77–131	0.30
M-43 (low potent) a)	153	0.13	81–124	0.29
b)	89	0.12	71–141	0.33
Relaxin Reference Standard W1164-A, Lot 8	123	1.12	77–130	0.31
As above, but with added non-pregnant sow ovarian tissue (2.5 mg./0.2 ml.)	139	0.97	80–125	0.26

DISCUSSION

The mouse interpubic ligament direct measurement technique provides a rapid, objective and practical method for the assay of relaxin preparations. Laboratory personnel rapidly develop proficiency in dissection and measurement of the pubic ligament. No unusual or elaborate equipment is required to conduct the assay.

All guinea pig palpation methods described (2, 3, 13) have a marked element of subjectivity. This factor becomes increasingly dangerous when investigators abandon the typical "mushy" or "foam rubber," late pregnancy-type of pubic symphysis as the prototype response, and attempt to "refine" the assay by classifying small "click" responses as positive. In addition to this, the sensitivity of individual animals in a colony varies from week to week (29), requiring careful randomization procedures. It is absolutely necessary that unknowns be coded, and that a standard be run concomitantly. Palpation of the symphysis pubis should be performed independently by at least two operators. When such precautions are taken reproducible and statistically valid results may be obtained when 60–120 guinea pigs are equally divided between two or three dose levels each of Standard and Unknown (13). Such numbers and precautions have not been reported as used by investigators other than Kroc *et al.* (13).

Inhibition of uterine motility *in vivo* or *in vitro* has served as a measure of relaxin activity. However, only Wiqvist and Paul (12) have published statistically meaningful data. These authors have drawn attention to the subjective element in their evaluation of responses. Kroc *et al.* (13) presented a similar *in vitro* assay in which the dosing schedule permitted more objective comparison of responses to Standard and Unknown. *In vivo* inhibition of uterine motility in guinea pigs described by Felton, Frieden and Bryant (9) yielded only qualitatively meaningful data in our laboratory (B. G. S., unpublished results). Felton and associates (9) have not published statistical evaluation of their method.

The mouse pubic symphysis type of assay offers a completely objective measure of response and has the additional advantages of simplicity and specificity. The advantages of direct measurement over x-ray techniques include speed and accuracy. X-ray, however, permits the use of pre-treatment control measurements in individual animals (7, 8), whereas separate control groups must be run in the direct measurement method. This is not a serious handicap, as the linear portion of the dose response curve is sufficiently long to permit the use of minimum doses of relaxin which still increase the interpubic ligament length by a factor of 2 versus estrogen-primed control mice.

The single dose of 5 μg. of estradiol cyclopentylpropionate for estrogen priming is simpler than the daily injections of 17β-estradiol used by Kliman *et al.* (7, 8, 17). In addition, greater sensitivity to relaxin is obtained using the repository estrogen rather than the free alcohol of estradiol. Kliman *et al.* (7) found it necessary to inject mice daily for 23 days with 17β-estradiol to obtain a sensitivity comparable to that found with the cyclopentylpropionate 7 days after a single injection. Single injections of other long-acting estrogens such as estradiol valerate or estradiol dipropionate have been used successfully for priming mice, but diethylstilbestrol was ineffective, even when used in depot form or administered daily in the drinking water (unpublished data).

The importance of a repository vehicle for relaxin injection in mice was demonstrated by Kliman and Greep (17). The selection of 1% L-390 (benzopurpurine 4B) as the vehicle of choice for routine assay was derived from an extensive screening of adjuvants and hyaluronidase inhibitors in our laboratories.

High and low potent and crude and purified relaxin extracts have been bioassayed against the same reference standard by the mouse and guinea pig methods (13, and data to be published elsewhere). The excellent agreement of potency estimates obtained indicates that both methods measure the same type of activity, and argues against the postulates of qualitatively different "mouse" and "guinea pig" relaxins (8). Kliman and Greep (17) have reached a similar conclusion. Data obtained by Wiqvist and Paul (12) and by Kroc *et al.* (13) suggest that "uterine relaxing factor" activity

closely parallels (and is probably identical with) pubic symphysis relaxin activity, when preparations are assayed against the same reference standard by mouse or guinea pig pubic symphysis assays, or by uterine motility inhibition methods. Reports of partial separation of "uterine relaxing factor" from relaxin activity (30, 31) are unconvincing because of failure of the authors to use a reference standard or present statistical analyses of their bioassay data.

Acknowledgments

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BIOLOGICAL ACTIVITY OF C-9 AND C-11 SUBSTITUTED DERIVATIVES OF Δ^4-ANDROSTENE-3,17-DIONE[1]

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ABSTRACT

The electrolyte, androgenic, and uterotrophic activities of 9α-chloro-11β-hydroxy-Δ^4-androstene-3,17-dione, 9α-bromo-11β-hydroxy-Δ^4-androstene-3,17-dione, 9α,11β-dihydroxy-Δ^4-androstene-3,17-dione, and 9β,11β-oxido-Δ^4-androstene-3,17-dione are reported. All four compounds showed sodium excreting activity at varying dose levels and of varying intensities. The chloro and oxido derivatives exhibited potassium excretion, the former being more active. All four steroids were essentially inactive as androgens when inuncted on the chick's comb while one steroid, the chloro derivative, showed a small but significant uterotrophic effect in the mouse.

WE HAVE described various biological effects of 9α-fluoro-11β-hydroxy-Δ^4-androstene-3,17-dione (1). Of these, the most striking was the ability of this steroid to retain sodium in adrenalectomized rats. Recently Selye (2) has demonstrated that this compound has life maintaining activity in the adrenalectomized rat. We have now had the opportunity to extend these studies as a result of the availability of additional related steroids including the 9α-chloro, 9α-bromo, and 9α-hydroxy derivatives of 11β-hydroxy-Δ^4-androstene-3,17-dione, as well as the 9β,11β-epoxide of Δ^4-androstene-3,17-dione.[2] These steroids have been studied for their effects on sodium and potassium balance in the adrenalectomized rat, androgenic activity in the chick, and uterotropic activity in the immature mouse.

METHODS

The electrolyte activity was measured by a modification of a previously described method (3). Adrenalectomized male rats weighing between 135–150 gm. received a sodium chloride load of 45 mg. administered intraperitoneally together with the test material administered subcutaneously. Urine was collected for 6 hours and sodium and potassium were determined by a flame photometer (Perkin-Elmer Model 52-C), using lithium as an internal standard. Each bioassay consisted of solvent controls, deoxycorti-

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[2] These compounds were generously supplied by Dr. Seymour Bernstein, Lederle Laboratories, Pearl River, N. Y.

osterone standards, and the unknown run in parallel. Androgenic activity was determined by application of the test material dissolved in oil or absolute alcohol directly to he male White Leghorn chick's comb (5). The test material, in parallel with the solvent ontrol and standards, was applied once daily in 0.05 ml. of solution for 7 days. Twenty-our hours after the last application, comb weights and body weights were determined. The results are expressed as the ratio of mg. comb/gm. of body weight. Uterotrophic activity was determined in immature Swiss Albino mice (4). The test material, standard, nd solvent control were administered once daily, in 0.1 ml. of corn oil, for three days. Uterine and body weights were determined 24 hours after the last injection and the results expressed as mg. uterus/gm. of body weight. At autopsy the animals were 24 to 27 days of age.

RESULTS

Data on the ability of the test materials to influence electrolyte metabolism are recorded in Tables 1–5. The steroid 9α,11β-dihydroxy-Δ^4-androstene-3,17-dione (Table 1) had no significant influence on potassium metabolism but a significantly ($P = 0.02$) increased excretion of sodium was found at the 25 μg. dose level. This effect was not observed at the higher dose levels of 50 and 100 μg. When 9β,11β-oxido-Δ^4-androstene-3,17-dione[3] was subjected to the same assay procedure, sodium excretion was observed at the 6, 25 and 50 μg. levels with a statistically ($P = 0.02$) significant result being recorded at the 6 μg. level. At the 100 μg. dosage level the sodium excretion remained unchanged. Potassium excretion remained unchanged except for a significantly increased excretion observed at the 50 μg. dose ($P = 0.01$) (Table 2). The results concerning 9α-chloro-11β-hydroxy-Δ^4-androstene-3,17-dione illustrate the fact that this steroid has no influence on sodium excretion while potassium excretion was observed at three different dosage levels (Table 3). At the 6 μg. dose in increase in urinary potassium was noted which was not statistically significant. In-

TABLE 1. THE INFLUENCE OF 9α,11β-DIHYDROXY-Δ^4-ANDROSTENE-3,17-DIONE ON SODIUM AND POTASSIUM METABOLISM

Steroid	Total dose, μg.	No. of adrenalectomized rats	Urinary excretion		
			Volume, ml. ±S.E.	Na, mg./100 gm./hr. ±S.E.	K, mg./100 gm./hr. ±S.E.
0	0	20	5.6 ± 0.4	1.58 ± 0.09	1.02 ± 0.06
Desoxycorticosterone	6	24	4.3 ± 0.3	1.39 ± 0.12	1.20 ± 0.12
	25	24	2.6 ± 0.2	0.78 ± 0.07	1.12 ± 0.10
9α,11β-Dihydroxy-Δ^4-androstene-3,17-dione	6	24	6.0 ± 0.5	2.07 ± 0.13	1.00 ± 0.50
	25	24	5.8 ± 0.5	2.57 ± 0.23	1.10 ± 0.29
	50	16	5.2 ± 0.4	1.89 ± 0.13	1.22 ± 0.11
	100	16	4.3 ± 0.5	1.74 ± 0.12	1.23 ± 0.07

[3] 9β,11β-Oxido-Δ^4-androstene-3,17-dione differs from the other C-9 and C-11 substituted steroids described in this paper in that the B/C ring fusion is cis rather than trans.

creasing the dosage to 25 μg. and 50 μg. resulted in significantly increased excretions ($P = 0.05$ and 0.01, respectively). Only one significant effect was found for 9α-bromo-11β-hydroxy-Δ^4-androstene-3,17-dione, this being increased excretion of sodium ($P = 0.01$) at the 50 μg. level (Table 4).

TABLE 2. THE INFLUENCE OF 9β,11β-OXIDO-Δ^4-ANDROSTENE-3,17-DIONE ON SODIUM AND POTASSIUM METABOLISM

Steroid	Total dose, μg.	No. of adrenal-ectomized rats	Urinary excretion		
			Volume, ml. ±S.E.	Na, mg./100 gm./hr. ±S.E.	K, mg./100 gm./hr. ±S.E.
0	0	16	6.1±0.4	1.58±0.11	1.00±0.07
Desoxycorticosterone	6	16	4.4±0.6	1.32±0.20	0.98±0.13
	25	14	2.6±0.5	0.79±0.16	0.88±0.17
9β,11β-Oxido-Δ^4-androstene-3,17-dione	6	16	6.9±0.4	2.08±0.16	0.99±0.08
	25	16	6.2±0.4	1.86±0.12	0.99±0.10
	50	8	6.4±1.3	2.03±0.21	1.32±0.06
	100	8	4.8±0.6	1.58±0.09	1.04±0.22

TABLE 3. THE INFLUENCE OF 9α-CHLORO-11β-HYDROXY-Δ^4-ANDROSTENE-3,17-DIONE ON SODIUM AND POTASSIUM METABOLISM

Steroid	Total dose, μg.	No. of adrenal-ectomized rats	Urinary excretion		
			Volume, ml. ±S.E.	Na, mg./100 gm./hr. ±S.E.	K, mg./100 gm./hr. ±S.E.
0	0	16	5.9±0.43	1.75±0.10	1.06±0.09
Desoxycorticosterone	6	16	4.1±0.6	1.34±0.12	1.35±0.13
	25	12	3.1±0.5	0.80±0.08	1.19±0.14
9α-Chloro-11β-hydroxy-Δ^4-androstene-3,17-dione	6	16	4.6±0.5	1.57±0.16	1.65±0.30
	25	16	4.4±0.4	1.60±0.15	1.58±0.21
	50	8	4.3±0.3	1.89±0.35	1.96±0.26
	100	6	5.0±0.12	1.40±0.17	1.17±0.18

TABLE 4. THE INFLUENCE OF 9α-BROMO-11β-HYDROXY-Δ^4-ANDROSTENE-3,17-DIONE ON SODIUM AND POTASSIUM METABOLISM

Steroid	Total dose, μg.	No. of adrenal-ectomized rats	Urinary excretion		
			Volume, ml. ±S.E.	Na, mg./100 gm./hr. ±S.E.	K, mg./100 gm./hr. ±S.E.
0	0	16	5.7±0.4	1.63±0.07	1.03±0.09
Desoxycorticosterone	6	16	3.9±0.5	1.36±0.06	1.21±0.15
	25	16	2.6±0.4	0.74±0.04	1.08±0.08
9α-Bromo-11β-hydroxy-Δ^4-androstene-3,17-dione	6	16	5.3±0.6	1.70±0.15	1.00±0.08
	25	16	3.5±0.2	1.65±0.13	1.10±0.13
	50	8	6.6±1.3	2.25±0.14	1.00±0.05
	100	6	5.7±0.10	1.62±0.10	0.94±0.09

TABLE 5. ANDROGENIC ACTIVITY OF C-9 AND C-11 SUBSTITUTED DERIVATIVES OF Δ^4-ANDROSTENE-3,17-DIONE
(Inunction in oil)

Steroids	Total dose, μg.	No. of chicks	Mean comb ratio ±S.E.
0	0	18	0.27 ±0.01
-Androstene-3,17-dione	40	12	0.61 ±0.05
	80	12	0.72 ±0.06
	160	12	0.93 ±0.06
β-Hydroxy-Δ^4-androstene-3,17-dione	40	12	0.33 ±0.02
	80	10	0.41 ±0.09
	160	10	0.49 ±0.04
,11β-Oxido-Δ^4-androstene-3,17-dione	40	11	0.27 ±0.02
	160	13	0.35 ±0.02
-Fluoro-11β-hydroxy-Δ^4-androstene-3,17-dione	40	12	0.34 ±0.02
	160	12	0.39 ±0.02
-Bromo-11β-hydroxy-Δ^4-androstene-3,17-dione	40	11	0.33 ±0.01
	160	10	0.35 ±0.02
α-Chloro-11β-hydroxy-Δ^4-androstene-3,17-dione	40	11	0.33 ±0.02
	160	12	0.32 ±0.01
α,11β-Dihydroxy-Δ^4-androstene-3,17-dione	40	12	0.38 ±0.02
	160	17	0.37 ±0.02

The data presented in Table 5 record decreased androgenic activities of each structural modification of Δ^4-androstene-3,17-dione. The activity of the parent compound is the greatest, the introduction of the 11β-hydroxy group significantly lowered the androgenic activity. Further substitution at carbon 9, either in the form of a halogen or oxygen, further decreased the androgenic activity when studied by direct application to the comb in an oil solution. Two of the compounds were studied by the inunction technic using absolute alcohol as the vehicle (Table 6). Here again the two compounds, 9α,11β-dihydroxy-Δ^4-androstene-3,17-dione and 9α-chloro-11β-hydroxy-Δ^4-androstene-3,17-dione, were found to be essentially inactive.

Uterotrophic activity of the C-9 substituted steroids, as measured by the increase in uterine weight, is presented in Table 7. The 9α-chloro-11β-hydroxy-Δ^4-androstene-3,17-dione showed a small but significantly higher activity than 11β-hydroxy-Δ^4-androstene-3,17-dione (1.09 ±0.06 compared to 1.34 ±0.09). The other steroids were essentially inactive.

DISCUSSION

The influence of C-9 substituent groups on biological activity of 11β-hydroxy-Δ^4-androstene-3,17-dione is summarized in Table 8. The most striking effects of these carbon 9 substituted C_{19} steroids are their influences on electrolyte metabolism. Neither Δ^4-androstene-3,17-dione nor the 11β-hydroxy derivative show any significant effects, but addition of the 9α-fluoro group produced a compound with high sodium retaining activity

TABLE 6. ANDROGENIC ACTIVITY OF C-9 AND C-11 SUBSTITUTED DERIVATIVES OF Δ^4-ANDROSTENE-3,17-DIONE
(Inunction in absolute alcohol)

Steroids	Total dose, μg.	No. of chicks	Mean comb ratio ± S.E.
0	0	19	0.32 ± 0.01
Androsterone	1	12	0.47 ± 0.03
	2	14	0.49 ± 0.04
	4	13	0.56 ± 0.04
	8	13	0.66 ± 0.04
	16	11	0.99 ± 0.07
9α-11β-Dihydroxy-Δ^4-androstene-3,17-dione	4	12	0.30 ± 0.02
	8	12	0.37 ± 0.02
	16	11	0.39 ± 0.02
	32	14	0.36 ± 0.02
9α-Chloro-11β-hydroxy-Δ^4-androstene-3,17-dione	4	4	0.33 ± 0.02
	8	8	0.34 ± 0.02
	16	16	0.35 ± 0.02
	32	32	0.34 ± 0.02

(1). The four compounds described in this report did not exhibit this sodium retaining effect. Actually, the chloro compound was inactive on sodium while the bromo substituted compound, the 9α,11β-dihydroxy and the 9β,11β-oxido, all exhibited sodium excretion at least at one dose tested. The three steroids which caused sodium excretion had in common an effective dose range while increasing the dose did not intensify the response but rather abolished the effect.

The 9α-chloro derivative was unique with respect to the potassium effect. At the three lowest levels increased potassium was excreted with the effect being abolished at the highest dose level.

TABLE 7. UTEROTROPHIC ACTIVITY OF C-9 AND C-11 SUBSTITUTED DERIVATIVES OF Δ^4-ANDROSTENE-3,17-DIONE

Steroid	Total dose, μg.	No. of mice	Mean uterine ratio ± S.E.
0	0	11	0.92 ± 0.05
Estrone	0.05	10	1.88 ± 0.09
	0.10	10	2.89 ± 0.13
	0.20	10	4.89 ± 0.30
11β-Hydroxy-Δ^4-androstene-3,17-dione	100	10	1.09 ± 0.06
9α-11β-Dihydroxy-Δ^4-androstene-3,17-dione	100	10	1.19 ± 0.09
9α-Chloro-11β-hydroxy-Δ^4-androstene-3,17-dione	100	10	1.34 ± 0.09
9β,11β-Epoxide-Δ^4-androstene-3,17-dione	100	10	0.95 ± 0.07
0	0	14	1.32 ± 0.07
Estrone	0.05	10	2.15 ± 0.14
	0.10	10	4.62 ± 0.25
9α-Bromo-11β-hydroxy-Δ^4-androstene-3,17-dione	100	10	1.14 ± 0.05

TABLE 8. MODIFICATIONS OF BIOLOGICAL ACTIVITY OF 11β-HYDROXY-Δ^4-ANDROSTENE-3,17-DIONE BY C-9 SUBSTITUENT GROUPS

New group at C-9	Effect on urinary		Androgenic activity (dose range, μg.)	Uterotrophic activity (dose μg.)
	Na	K		
Fluorine	Retention (6–25)	—	Decreased (40, 160, 320)	Increased (50)
Chlorine	No Effect (6, 25, 50, 100)	Excretion (25–50)	Decreased (40, 160)	Increased (100)
Bromine	Excretion (50)	No Effect (6, 25, 50, 100)	Decreased (40, 160)	Increased (100)
Hydroxy	Excretion (25)	No Effect (6, 25, 50, 100)	Decreased (40, 80, 160)	Increased (100)
Oxido	Excretion (6)	Excretion (50)	Decreased (40, 80, 160)	Increased (100)

Modification of the 11β-hydroxy-Δ^4-androstene-3,17-dione molecule by the 9-halogen or 9-oxygen function reduced the androgenicity. These observations are in keeping with the studies on 9α-fluoro-11β-hydroxy-Δ^4-androstene-3,17-dione previously reported (1). Uterotrophic activity, on the other hand, was maximally intensified by the introduction of the fluorine at C-9. The steroid with a chlorine substituent at C-9 was active on the uterus but the bromo, the hydroxy, and the oxido derivatives were inactive at the dose levels studied.

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NOTES AND COMMENTS

THE CORPUS LUTEUM-PITUITARY RELATIONSHIP: ON THE REPORTS THAT OXYTOCIN STIMULATES THE SECRETION OF LUTEOTROPHIN[1]

ABSTRACT

Injections of oxytocin, vasopressin or saline were made under a variety of conditions into separate groups of adult cycling female rats. Only 8 prolonged cycles (out of a total of 162), bearing no specific relation to oxytocin treatment, were observed during or following treatment. Lactating rats, deprived of their litters and subjected to uterine trauma on the 5th or 7th day postpartum, almost uniformly formed deciduomata, regardless of oxytocin treatment; when litters were removed on day 2, and the uterus traumatized on day 6, 50% formed deciduomata, regardless of oxytocin treatment. In the light of these and other results the theory that oxytocin stimulates LTH secretion was considered to be invalid.

That oxytocin stimulates luteotrophin (LTH)[2] secretion has been suggested by the following. Milk secretion was maintained for 9 days (but not for 12) in oxytocin, but not saline-treated post-parturient rats deprived of their litters on day 4 postpartum (1). Certain hypothalamic lesions in lactating rats were associated with a "defect in milk secretion"; this was corrected by oxytocin treatment (2). Treatment of cycling rats with oxytocin resulted in pseudopregnancy (3). Deciduomata formed in oxytocin, but not saline-treated post-parturient rats deprived of their litters and subjected to uterine trauma (4). The experiments described in this note attempted to confirm some of these findings.

EXPERIMENTS AND RESULTS

Induction of pseudopregnancy. Separate groups of adult cycling Sprague-Dawley female rats were injected with oxytocin, vasopressin or saline under the conditions described in Table 1. Pseudopregnancy-like cycles occurred infrequently, and in no specific relation to oxytocin treatment (Table 1).

Formation of deciduomata in post-parturient rats. The conditions of oxytocin treatment, litter removal, and uterine trauma are outlined in Table 2. The incidence of deciduoma formation was practically the same in the oxytocin-treated rats and their controls (Table 2), although some of the oxytocin-treated rats may have formed somewhat larger deciduomata than did the controls.

DISCUSSION

The experiments summarized in Table 1 indicate that oxytocin does not elicit the luteotrophic endpoints of LTH secretion. McCann and Friedman in rats (5), and Folley in mice (6) have also been unable to demonstrate this effect (3).

The experiments reported in Table 2 suggest that under the conditions of Stutinsky's experiment (4), LTH is secreted "spontaneously." The finding that a pseudopregnancy-like diestrus is shown by almost 50% of post-parturient rats deprived of their litters as early as day 2 postpartum (7) supports this suggestion. Work in progress indicates that this "spontaneous" LTH secretion is actually dependent on ovarian activity, and cannot be maintained by oxytocin (8). It has been found that, in *oxytocin-treated* post-parturient rats deprived of their litters, milk secretion can be maintained for at least 28 days in

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[2] See the introduction to (7) for the definition and justification of this term.

TABLE 1. EFFECTS OF OXYTOCIN, VASOPRESSIN, OR SALINE INJECTIONS ON THE ESTROUS CYCLES OF ADULT, INTACT, CYCLING RATS

Material	Injections[1]			No. of rats	Results	
	Amount (I.U. or cc.)	Daily schedule	Duration in days		No. of cycles observed during or following treatment	No. of prolonged cycles
Oxytocin	0.83	Every 4 hrs.	16	7	20	1
Saline	0.25	Every 4 hrs.	16	6	18	0
Oxytocin	2.5	Once, in proestrus only		4	16	0
	5.0	Once, in proestrus only		6	24	0
Vasopressin	2.5	Once, in proestrus only		3	12	1[2]
	5.0	Once, in proestrus only		6	24	0
Oxytocin	5.0	Once daily	9	2	8	0
	5.0	Once daily	23	2	8	2[3]
Vasopressin	5.0	Once daily	9	2	8	0
	5.0	Once daily	23	2	8	2[3]
Saline	0.5	Once daily	9	2	8	0
	1.0	Once daily	23	2	8	2[4]
Totals	—	—	—	41	162	8

[1] All injections were given by the intraperitoneal route, except in the first 2 experiments, in which they were given subcutaneously. In the first experiment each oxytocin injection was made in a volume of 0.25 cc. In the others, the concentration of oxytocin and of vasopressin was 10.0 I.U./cc.
[2] Immediately followed injection.
[3] One occurred immediately; the other was the second cycle in the other rat.
[4] First cycle in each rat.

TABLE 2. THE EFFECT OF OXYTOCIN ON THE FORMATION OF DECIDUOMATA IN POST-PARTURIENT RATS DEPRIVED OF THEIR LITTERS

Type of treatment and duration[1]	Time of removal of litter (day postpartum)	Time of uterine traumatization (day postpartum)	No. of rats	Deciduoma responses[4]				
				0	+	++	+++	++++
Oxytocin: 0.5 I.U. 5×/day×5 days, SC[2]	5	5	2	—	—	—	—	2
Oxytocin: 3.0 I.U. ×1, IP[3]	5	5	4	—	—	—	1	3
None	5	5	7	1	1	—	3	2
Oxytocin: 3.0 I.U. ×1, IP[3]	2	6	4	2	1	1	—	—
None	2	6	8	4	2	—	2	—
None	7	7	11	3	1	—	2	5

[1] In all cases oxytocin treatment was given or begun on the day of litter removal.
[2] Subcutaneously.
[3] Intraperitoneally.
[4] These gradings conform roughly to the following weights for a single traumatized uterine horn: 0 = 100–250 mg.; + = 350–400 mg.; ++ = 400–500 mg.; +++ = 500–600 mg.; ++++ = over 600 mg. The uteri were examined 5 days after traumatization.

intact rats treated with progesterone, and that castration without progesterone treatment reduced the duration of milk secretion to about 10 days or less. Castrates treated with progesterone, however, or bearing a homotransplanted pituitary beneath the kidney capsule but not given progesterone, were still secreting milk after at least 15 days of treatment (8). These results indicate that oxytocin does not elicit the lactogenic endpoints of LTH secretion, and suggest instead that the maintenance of milk secretion by oxytocin (1, 2) may be due to a local effect on the mammary gland, probably through its ability to discharge milk from the aveoli (9, 10). This could explain the results of McCann, Mack and Gale (2), since in another experiment McCann and Friedman (5) found that rats bearing the same type of hypothalamic lesions secreted LTH spontaneously, in a manner reminiscent of the transplanted pituitary (11, 12, 13).

Since the incidence of pseudopregnancies following early litter removal in our colony is only about 50%, it is difficult to explain the fact that practically *all* such oxytocin-treated rats (1) or those subjected to uterine trauma (Table 2) give evidence of continued LTH secretion. Perhaps uterine trauma permitted the maintenance of pseudopregnancy through a mechanism similar to that which prolongs the pseudopregnancy in rats bearing deciduomata (14, 15). In the oxytocin-treated rats, the distention of the ducts with milk (10), produced by oxytocin's "letdown" effect (9), may have served as a mild local irritant to the nipple area that was enough to maintain LTH secretion in *all* rats.

A final point is this. Benson and Folley's oxytocin-treated rats showed vaginal mucification, while their saline-treated controls showed atrophic vaginal mucosae (1). However, if their experimental rats secreted progesterone, *in the absence of estrogen*, their vaginal mucosae would not have been different from the controls. This means a) that the controls could also have secreted progesterone, and b) that oxytocin caused vaginal mucification through some effect other than the stimulation of LTH secretion. The latter possibility has been confirmed by the finding of marked vaginal mucification in oxytocin treated hypophysectomized rats bearing autotransplanted pituitaries (7).

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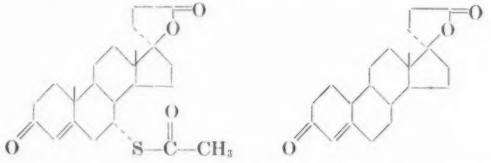
BLOCKING THE RENAL ELECTROLYTE EFFECTS OF MINERALOCORTICOIDS WITH AN ORALLY ACTIVE STEROIDAL SPIROLACTONE

ABSTRACT

Aldactone, or 3-(3-oxo-7α-acetylthio-17β-hydroxy-4-androsten-17α-yl)propionic acid γ-lactone, blocked the urinary electrolyte effects of desoxycorticosterone acetate in rats, by simultaneously reversing sodium retention and potassium loss. The compound was $\frac{1}{4}$ as active parenterally and 5 times as active orally relative to an early steroidal spirolactone, SC-8109 or 3-(3-oxo-17β-hydroxy-19-nor-4-androsten-17α-yl)propionic acid γ-lactone, which has received clinical trials in edematous states and other conditions characterized by aldosteronism. Aldactone was approximately as active orally as parenterally, and it also reversed the urinary electrolyte effects of aldosterone and hydrocortisone. The compound was inactive when given alone. All evidence favors the view that the compound affects electrolyte excretion by competitive inhibition with aldosterone-like steroids at the renal level. The compound did not show cortisone-like, androgenic, anabolic, estrogenic or progestational activity; it was similarly inactive in inhibiting the effects of standard steroids in the endocrinologic tests. The studies indicate that the compound possesses an oral potency which may be useful for the treatment of edema.

Recent reports described steroidal spirolactones (1, 2), which possessed the ability to block the urinary electrolyte effects of aldosterone and desoxycorticosterone in animals (3–6). Following administration of these compounds in cardiac and nephrotic patients with edema, Liddle reported sodium and water diuresis and potassium retention (4, 7), and the electrolyte effects in man as in the animal studies were ascribed to the antagonism to aldosterone-like steroids. Thus, the clinical findings suggested an important etiological significance of aldosterone in these edematous states and a potential therapeutic utility of aldosterone-blocking agents in edema. However, the early examples of the steroidal spirolactones showed clinical effects at relatively large oral doses (7–14). It was of interest, therefore, that further search yielded another spirolactone with interesting oral activity. The present report will describe the blocking ability of this compound, SC-9420 (15) or Aldactone,[1] relative to that of SC-8109 (1), an early example and a subject of recent reports (16–31). The structures of the 2 compounds are shown below.



SC-9420 (left): Aldactone or 3-(3-oxo-7α-acetylthio-17β-hydroxy-4-androsten-17α-yl)propionic acid γ-lactone.
SC-8109 (right): 3-(3-oxo-17β-hydroxy-19-nor-4-androsten-17α-yl)-propionic acid γ-lactone.

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[1] Registered trademark of G. D. Searle and Co. for spironolactone.

METHODS

Male Sprague-Dawley rats (150–200 gm.) were adrenalectomized and maintained overnight with sucrose cubes and tap water. Approximately 24 hours postoperatively, all animals were injected subcutaneously with 2.5 cc. 0.85% sodium chloride solution and with 12 μg. of desoxycorticosterone acetate (DCA) in 0.1 cc. Mazola oil. Aldactone was given additionally either as a subcutaneous injection or an oral dose in 0.5 cc. oil in groups of 6–10 animals. The bladder of the animals was emptied by application of a slight pressure with the fingers before placing the animals in metabolism cages. Voiding was again induced at the end of a 4-hour correction period for complete recovery of urine. Pooled specimens were analyzed for sodium and potassium content after dilution to a definite mark with water. The concentration values representing measures of total sodium and potassium were then used for the computation of the log $(Na \times 10)/K$ ratio.[2] In this method, a 12 μg. dose of DCA causes reduction of the ratio of Na/K by promoting sodium retention and potassium loss. Blocking activity was measured by reversal of the Na/K effect of DCA. Log transformation of the Na/K values gave in our standardization tests responses with relatively constant standard error values at different levels of DCA. From this standpoint, the adjustment provided a statistically useful response index for the evaluation of DCA-blocking activity. The proportion of the standard dose of DCA (as % block) inhibited by Aldactone was determined at different dosage levels by comparison with a DCA calibration curve. The dose of Aldactone required for 50% inhibition, i.e., for reduction of the Na/K effect of 12 μg. of DCA to that obtained with a 6 μg. dose, was then determined by interpolation using cumulated dose-response data. The dose for this degree of inhibition was defined arbitrarily as the median effective dose (MED).

The ability of Aldactone to block the urinary electrolyte effects of other steroids was also studied in similar animals. In one instance, *d,l*-aldosterone-21-monoacetate[3] in 0.1 cc. 20% ethanol-saline (20/80 by volume) was given alone and in combination with Aldactone in oil. A similar test was undertaken with hydrocortisone as the mineralocorticoid in 0.5 cc. oil. Groups of adrenalectomized animals were injected subcutaneously with Aldactone in the absence of DCA to study the possibility of the compound having a direct effect on Na/K, i.e., an action opposite to that of DCA. The diuretic effects of the compound when given alone were assessed in intact animals. The spirolactone in oil was injected subcutaneously into saline-loaded rats (275–400 gm. body weight), and the effects on sodium and water in 5 hours evaluated in terms of a standard dose of urea (32). Diuretic activity was also determined in trained female mongrel dogs weighing 10 to 20 kg. A 5-hour sample of urine was collected by catheterization after the subcutaenous injection of Aldactone in oil, and the urinary measurements compared with those of the control responses for the dogs. The compound also received various tests for cortisone-like, anabolic, androgenic, estrogenic and progestational activity, as well as tests for possible inhibitory effects on action of standard endocrine steroids. All analyses for sodium and potassium concentration were done with the Beckman flame photometer.

RESULTS

The DCA-blocking effects of various doses of Aldactone are summarized in Table 1. The data demonstrate that DCA alone reduced the urinary ratio of Na/K by causing sodium retention and potassium loss. In the presence of increasing doses of Aldactone, however, DCA was progressively less effective on Na/K. In general, blocking appeared to have resulted from the simultaneous reversal of sodium and potassium effects. Fig-

[2] Multiplication of Na concentration by 10 eliminated negative log Na/K values.

[3] Kindly supplied by Dr. R. T. Hill of the Endocrinology Study Section, National Institutes of Health, Bethesda, Maryland; the racemic preparation was approximately 40 times as potent as DCA (by weight) when evaluated by the Na/K response.

TABLE 1. SUMMARY OF DATA DEMONSTRATING THE DCA-BLOCKING EFFECTS OF ALDACTONE AFTER SUBCUTANEOUS ADMINISTRATION IN ADRENALECTOMIZED RATS

Treatment/rat		Number test animals	Mean log values		
DCA, μg.	Aldactone, mg.		Na	K	(Na×10)/K
—	—	65	0.78	0.45	1.34
12	—	66	0.36	0.62	0.77
12	0.1	10	0.46*	0.56*	0.91*
12	0.3	10	0.45	0.53*	0.91*
12	0.4	10	0.61*	0.55*	1.06*
12	0.8	10	0.73*	0.61	1.12*
12	1.2	8	0.59*	0.56*	1.04*
12	1.6	10	0.77*	0.57*	1.20*
12	2.4	10	0.70*	0.55*	1.16*
12	4.8	6	0.80*	0.63	1.17*

* $P \leq 0.05$, relative to the DCA controls, one-tail test; estimated variances ($\times 10^{-4}$) per rat response = 69.1, 18.8 and 43.1 for log Na, K and (Na×10)/K, respectively; Na and K = log mM/L values after dilution of sample/rat/4 hours to 25 cc. with water.

ure 1 demonstrates the blocking ability of Aldactone, in terms of the proportion of the standard dose of DCA inhibited by the compound. By interpolation, it was found that an injection of 0.33 mg. Aldactone/rat was required for 50% inhibition. Orally, the data describe an MED value of 0.48 mg., which deviation from the parenteral MED represents a $P > 0.5$ at 60 degrees of freedom. Thus, the compound is as effective orally as parenterally. The difference in slope of the curves by the 2 treatments was nonsignificant, to indicate that the same oral/parenteral dosage ratios would be expected at levels other than 50% block. In general, there was a large variability in response of the measurements as might be expected with the analysis of pooled data. The variance with oral dosage was significantly greater than that obtained with the injections ($P < 0.05$). For purposes of comparison the estimates of the MED for Aldactone are summarized in Table 2 together with those of SC-8109, the 19-nor-compound. The results show that Aldactone possesses one fourth the potency of SC-8109 given parenterally; the difference of the MED values is significant ($P < 0.05$). Orally, SC-8109 showed an MED value of

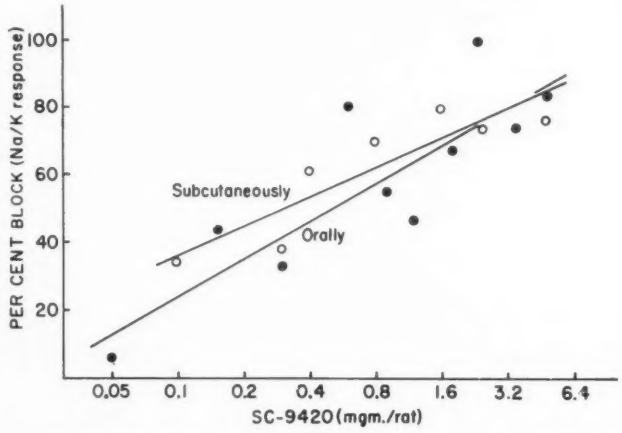

FIG. 1. Action of Aldactone (SC-9420) parenterally (○) and orally (●) in blocking effects of desoxycorticosterone acetate on the urinary ratio of Na/K (b = 30.7 and 37.5% block/10-fold range of log doses with subcutaneous and oral treatment, respectively).

TABLE 2. RELATIVE POTENCIES OF ALDACTONE AND SC-8109 GIVEN PARENTERALLY AND ORALLY IN BLOCKING THE URINARY Na/K EFFECTS OF DESOXYCORTICOSTERONE ACETATE IN ADRENALECTOMIZED RATS

Route of administration	Compound	Number test animals	MED	Relative potency
Subcutaneously	SC-8109	208	0.08 (0.035–0.220)	1
	Aldactone	74	0.33 (0.18–0.61)	0.27
Orally	SC-8109	84	2.45 (1.28–4.69)	1
	Aldactone	122	0.48 (0.19–1.23)	5.1

MED = median effective dose in mg./rat required for a 50% block or the reduction of the log Na/K effect of 12 to 6 μg. of DCA; values in parentheses = 95% fiducial limits; difference in MED values = $P < 0.05$ subcutaneously and $P < 0.01$ orally.

2.45 mg./rat, and Aldactone an MED of 0.48 mg. Thus, by presumably better absorption in the gastrointestinal tract, Aldactone showed an oral potency of approximately 5 relative to that of SC-8109 (MED SC-8109/MED Aldactone = 2.45/0.48); the difference in potency by the oral route was highly significant ($P < 0.01$). The slopes of the dose-response curves for the two compounds, either parenterally or orally, were not different.[4]

Table 3 demonstrates the ability of the compound to block the effects of racemic aldosterone-21-monoacetate. Aldosterone alone produced a low value of Na/K. Its effect on Na/K, however, was significantly reversed in the presence of the steroidal spirolactone, with blocking resulting from the simultaneous antagonism of sodium retention and potassium loss. The compound also blocked the effects of hydrocortisone as shown in the same table. In this instance, reversal of the Na/K response was obtained primarily from changes of sodium excretion.

The electrolyte effects of Aldactone given alone in the adrenalectomized rats are

TABLE 3. ACTION OF ALDACTONE IN BLOCKING THE URINARY ELECTROLYTE EFFECTS OF ALDOSTERONE AND HYDROCORTISONE IN ADRENALECTOMIZED RATS

Treatment/rat		Number of measurements	Mean log response		
Mineralocorticoid	Aldactone		Na	K	(Na×10)/K
0.9 μg. Aldosterone	—	4	0.82	1.03	0.80
0.9 μg. Aldosterone	0.2 mg.	2	0.87	0.98	0.89
0.9 μg. Aldosterone	0.4 mg.	2	0.97*	0.95	1.02*
0.9 μg. Aldosterone	0.8 mg.	2	1.03*	0.92*	1.11*
400 μg. Hydrocortisone	—	4	1.08	1.30	0.78
400 μg. Hydrocortisone	0.2 mg.	2	1.24*	1.32	0.93*
400 μg. Hydrocortisone	0.4 mg.	2	1.27*	1.22	1.05*
400 μg. Hydrocortisone	0.8 mg.	2	1.37*	1.23	1.14*

* $P \leq 0.05$, relative to controls; least significant change (P of 0.05) = +0.15 for Na, −0.10 for K and +0.13 for Na/K; 4 rats/measurement; Na and K = log mM/L values after dilution of urinary sample/4 rats/4 hours to 50 cc. with water.

[4] Variance ratios, F, were 0.037 and 0.89 for subcutaneous and oral treatment, respectively; these values were far below the limit of 4.5 required for significance ($P = 0.05$, $n_1 = 1$ and $n_2 = 15$–16).

ummarized in Table 4. None of the Na/K responses with treatment fell outside of the imits for a 1 in 20 chance observation. There was a retention of potassium with the 0.26 g. dose ($P < 0.05$); the significance of this response is unclear, inasmuch as no effect was en with the 7.8 mg. dose. These data demonstrate that Aldactone does not directly ffect sodium and Na/K over a wide range of doses; nor is the compound capable of roducing consistent effects opposite to those of DCA. The compound was not effective producing statistically significant diuresis in saline-loaded intact rats at a dose of 12 g./kg. No significant effects were found on urinary sodium, potassium and volume with e same dose of the compound in dogs. Contrastingly, acetazolamide, chlorothiazide, isometradine and the mercurials are effective in the dogs; all except the mercurials e similarly active in the rats.

Aldactone did not show significant endocrine or anti-hormonal properties in various sts. 1) The compound given at a 10 mg. total dose subcutaneously was inactive on

TABLE 4. EFFECTS OF ALDACTONE WHEN GIVEN ALONE ON URINARY ELECTROLYTE EXCRETION IN ADRENALECTOMIZED RATS

Treatment/rat	Number of measurements	Mean log response		
		Na	K	(Na×10)/K
—	4	1.37	0.86	1.51
0.26 mg. Aldactone	3	1.34	0.74*	1.59
7.8 mg. Aldactone	3	1.26	0.84	1.43

* $P < 0.05$, relative to controls; least significant change (P of 0.05) = ±0.15 for Na, ±0.11 for K and ±0.13 for Na/K; 4 rats/measurement; Na and K = log mM/L values after dilution of urinary sample/4 rats/4 hours to 50 cc. with water.

liver glycogen deposition in adrenalectomized rats. The same dose failed to inhibit effects of 0.5 mg. of cortisone acetate. 2) In a 2-day test in adrenalectomized rats, Aldactone at 5 mg./day subcutaneously did not affect granuloma formation of cotton pellet implants, nor did the same dose block the anti-inflammatory effects of hydrocortisone (1 mg./day). 3) Aldactone did not enhance Coxsackie virus infection in mice when 2.5 mg. were injected subcutaneously each day over 6 days. Likewise, the compound was ineffective in promoting the infection with streptococci at the same daily dose over 5 days. Cortisone is active in both tests at a dose of 0.25 mg. 4) The weight of the seminal vesicles and prostate in castrated rats was not affected following intramuscular injection of Aldactone at a total dose of 5 mg. for 7 days. Anabolic activity was not found with the same dose, as judged from the levator ani response. Given in combination with 0.5 mg. testosterone propionate, Aldactone did not inhibit anabolic activity or the weight stimulation of the male accessory organs. 5) Uterine growth was not stimulated in immature mice following treatment with a total dose of 1,000 μg. of Aldactone subcutaneously over 3 days. The uterotrophic effects of 0.3 μg. of estrone were not inhibited with Aldactone over the dosage range of 10–300 μg. 6) Progestational activity was determined in immature rabbits which were primed with injections of estradiol (Clauberg assay). Aldactone given at a daily dose of 0.5 and 1.0 mg. subcutaneously did not produce endometrial gland proliferation. The effects of 0.1 mg. progesterone subcutaneously were not inhibited with the large dose of the compound.

DISCUSSION

Our data suggest that Aldactone exerts effects on electrolytes by competing with aldosterone-like steroids for receptor sites in target tissues. It reverses the effects of DCA, aldosterone and hydrocortisone to suggest that the target tissues for electrolyte

manifestations are common to many steroids and to the steroidal spirolactone. Aldactone does not appear to affect ion excretion directly in a manner opposite to the effect of DCA, thus ruling out physiological antagonism as a mechanism of action. Thus, it is not surprising that the spirolactone given alone also failed to produce sodium and water diuresis in intact rats and dogs, in which presumably only small amounts of aldosterone are found as suggested by several studies (33–36). The inhibition is presumed to be at the kidney level (20, 21), the generally accepted site of action of mineralocorticoids for effects on sodium and potassium excretion (37–38).

Aldactone is of interest as a blocker of DCA because of its favorable oral potency in laboratory animals. It is approximately 5 times as potent orally as SC-8109, a 19-nor-steroidal spirolactone, and is equally as effective by the oral or parenteral route of administration. By comparison of effects obtained with the parent compound, SC-5233 or 3-(3-oxo-17β-hydroxy-4-androsten-17α-yl)propionic acid γ-lactone (1, 3–6), it appears that the acetylthio-modification at carbon 7 had little effect on parenteral activity. The MED of SC-5233 is estimated to be 0.22 mg. (95% limits = 0.088–0.55), as constrasted with the MED of 0.33 mg. for Aldactone (Table 2). For all practical purposes, these values of MED can be considered the same (P = approximately 0.5 for difference between the MEDs). If the relative potencies are considered in terms of molecular proportions, rather than in terms of weight, there would be a closer approximation of the MED values for the 2 compounds.[5] The MED of SC-5233 by oral treatment is estimated to be a massive dose of 19.2 mg./rat. By this route, it appears that the 7α-acetylthio-radical increased activity by approximately 40-fold on a weight dosage basis; activity would be increased by about 50-fold by molecular proportions. Results of other studies show that Aldactone possesses a relatively specific effect in inhibiting aldosterone-like action. It was inactive in inhibiting the effects of standard steroids in tests for cortisone-like, anabolic, androgenic, estrogenic or progestational activity. The compound was similarly inactive when given alone in the endocrinologic tests.

In addition, the aldosterone- and desoxycorticosterone-blocking ability of Aldactone is of interest because of a possible etiological significance of aldosterone in edema (40–42). Since the initial observations (4, 7), clinical reports have appeared confirming effectiveness of the steroidal spirolactones in edematous states (10, 12, 13, 16, 23, 26) and in various states of aldosteronism (8, 13, 20, 23, 24, 28). In general, the clinical findings appear to support 2 points: a) competitive inhibition with aldosterone-like steroids as the mechanism of effect of the steroidal spirolactones to show good agreement with our laboratory data (cf. 9, 11), and b) an important role of aldosterone in edema (cf. 42–45). Our laboratory data indicate that Aldactone would be about 5 times as potent as the 19-nor-compound, SC-8109 (3, 11, 13, 14, 18, 20, 24, 46), when administered orally in man. Early studies show that Aldactone is a useful oral agent for the treatment of edema (25, 47–54).

Acknowledgment

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[5] Molecular weights of Aldactone and SC-5233 are 416.6 and 342.5, respectively.

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THE EFFECT OF GLUCAGON ON HINDQUARTER BLOOD FLOW IN THE DOG[1]

ABSTRACT

Direct measurement of the hindquarter blood flow was obtained in an acute preparation in which the inferior vena cava was cannulated just distal to the renal veins. Following the intravenous administration of .02 mg. glucagon/kg., to dogs in a postabsorptive state, there was a marked decrease in hindquarter blood flow. A mean decrease to 52% of control values occurred between 10 and 20 minutes after glucagon. This effect was not observed in a dog fasted 5 days. Administration of glucose, in amounts roughly comparable to the amount of glucose released by the liver with this dose of glucagon, produced a transient increase in hindquarter flow. These findings, together with previously reported findings of increased hepatic blood flow, suggest a redistribution of cardiac output following glucagon.

Since its crystallization in 1953, glucagon has been studied extensively, its physical properties characterized, its structure analyzed and its biologic effects described (1–5). In reviewing the literature on glucagon, Van Itallie (1) found that no hemodynamic effect had been observed at that time (1956); Bondy, *et al.*, (6) reported that there was no change in peripheral blood flow after glucagon administration in man.

We have previously reported an increase in hepatic blood flow after intravenous administration of crystalline glucagon (7). The mean hepatic blood flow doubled within 10 to 20 minutes, and this increase was diminished or absent when glycogen stores had been depleted after 3 to 5 days of fasting (8). In the present study a hindquarter blood flow method is described preliminary to measurement of regional metabolism of this peripheral area. Furthermore, this method was employed to determine whether the

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increase in hepatic blood flow was associated with a concomitant change in peripheral blood flow.

The rate of blood flow in the inferior vena cava just distal to the renal vessels was measured in this study and used as an index of the peripheral blood flow. The venous blood flow in the distal vena cava under steady state conditions is equal to the blood flow of the terminal aorta and was taken to represent the blood flow to the hindquarters.

METHODS AND MATERIALS

Mongrel dogs of both sexes weighing 16 to 23 kilograms were used in these experiments. The dogs were anesthetized with sodium thiopental intravenously; respiratory rate and depth was controlled with a Harvard respiration pump through an endotracheal tube. A large (6 mm. internal diameter) cannula was inserted into the right jugular vein; the right carotid artery was also cannulated for arterial blood sampling and for monitoring arterial blood pressure. Heparin (2 mg./kg.) was given. The inferior vena cava just distal to the renal veins was exposed through a lower abdominal midline incision. The lumbar veins distal to the proposed cannulation site were tied and the distal cava was cannulated with large plastic tubing (9 mm. internal diameter). This cannula led to an extracorporeal reservoir of donor dog blood which was connected to a Sigmamotor pump that returned the blood to the right jugular vein via the previously placed cannula. The extracorporeal system was set up before the abdominal operation so that immediately upon cannulization of the vena cava the extracorporeal circuit could be established.

Venous flow from the hindquarters was measured directly by diverting the caval blood from the reservoir into a graduated cylinder for a given, timed period. A sufficient extracorporeal reservoir was provided so that measurements of flow could be obtained without disturbing the animal's blood volume, arterial blood pressure or hindquarter blood flow. The sampled blood was replaced with equal quantities of heparinized dog blood.

Using this preparation the effect of glucagon on the venous blood flow from the hindquarters was studied. The experimental animals were studied 18 to 24 hours after their last meal; one dog was fasted for 5 days prior to flow determinations. After 6 to 12 baseline flow measurements were taken, glucagon 0.02 mg./kg. was given as a single intravenous injection. Hindquarter blood flow determinations were repeated at 2 to 10 minute intervals until flow returned to baseline or near baseline levels. Variations in flow occurring between actual measurements were readily observed by changes in reservoir volume. Flow measurements were taken at definite intervals and when changes in flow required adjustment of pump speed.

The glucagon effect on hindquarter blood flow was compared with the effect of glucose or saline. The single intravenous injection of 0.4 mg. glucagon was found to release approximately 9 gm. of glucose in the first hour after injection (or a net increase of 6 gm.) (8). Ten grams of glucose, an amount roughly comparable to that released by the liver after glucagon, was administered as a 50% aqueous solution both intra-arterially (in the carotid cannula) and intravenously (into the femoral vein). As a further control, 10 gm. of glucose as a 5% aqueous solution was infused into the jugular vein. This was compared with the infusion of an equal volume (200 cc.) of isotonic saline into the jugular vein. These glucose and saline infusions were injected over a period of 1 to 2 minutes.

RESULTS

The results of the series of experiments are summarized in Figure 1. There was a marked decrease in hindquarter blood flow after intravenously administered glucagon. This flow decrease occurred rather promptly and reached its nadir 10 to 20 minutes after the injection of glucagon. There was a return to control or near control values by 45 minutes following glucagon administration. The mean hindquarter blood flow at its lowest point was 52% of control values. No significant change in arterial blood pressure was observed.

The dog which had been starved for 5 days was similarly studied and in this instance there was a minimal flow change (Fig. 2). Coincident with this, a minute rise in arterial blood sugar was observed (from a control value of 102 mg./100 cc. to 114 mg./100 cc., 10 minutes after glucagon). This lack of effect of glucagon corroborated the lack of effect of glucagon on hepatic blood flow and hepatic glucose output previously reported in the fasted animal (8).

Intravenous administration of 10 gm. glucose as a 5% solution, or as a 50% solution, produced an increase in hindquarter flow which, though pronounced, was evanescent. Similarly the addition of 200 cc. of saline produced a temporary increase in hindquarter flow (Fig. 3).

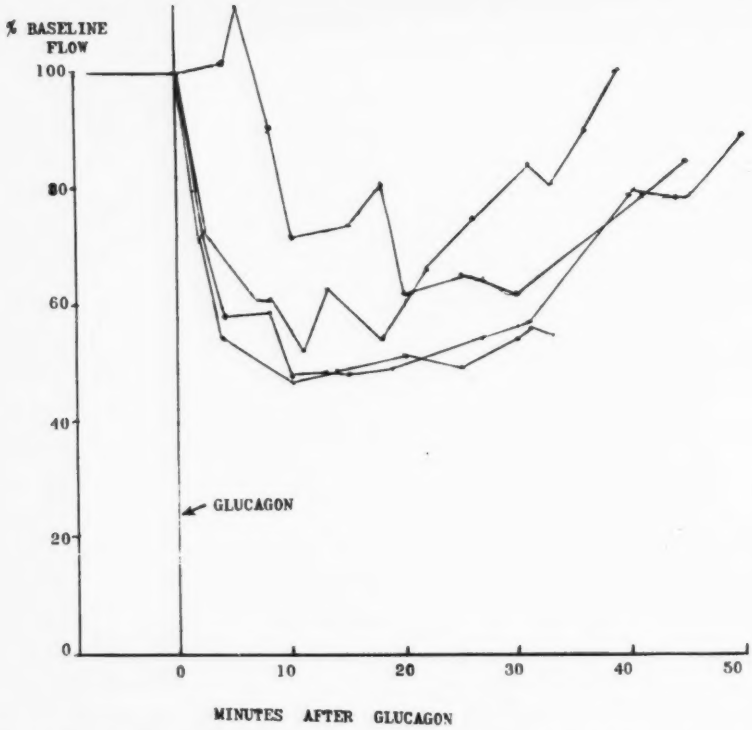

FIG. 1. Hindquarter blood flow as a function of time after administration of crystalline glucagon .02 mg./kg. The rate of blood flow of individual dogs is represented as a percentage of the mean control value.

DISCUSSION

These studies indicate that in dogs there is a decrease in hindquarter blood flow following the intravenous administration of glucagon. Several questions arise in the interpretation of these results: are the hindquarter blood flow changes due to glucose released by glucagon; to a direct action of the hormone itself; or do they occur as a consequence of increased flow to the liver?

The administration of glucose (in amounts comparable to that released by the liver after glucagon) produced an increase rather than a decrease in hindquarter blood flow in this preparation. This occurred with both isotonic and with concentrated glucose. The increase in flow in either case was sudden, marked, and shortlived; these responses paralleled those occurring after the addition of 200 cc. of isotonic saline. Thus, the responses to glucose and saline administration are regarded as due largely to the sudden expansion of the circulating plasma volume in the system.

The hindquarter blood flow change followed glucagon administration in the same time relationship as the hepatic blood flow alteration previously reported; both flow changes reach their maximum deviations from control values in 10 to 20 minutes. They differed, however, in the direction of the flow change. Whereas the hepatic blood flow

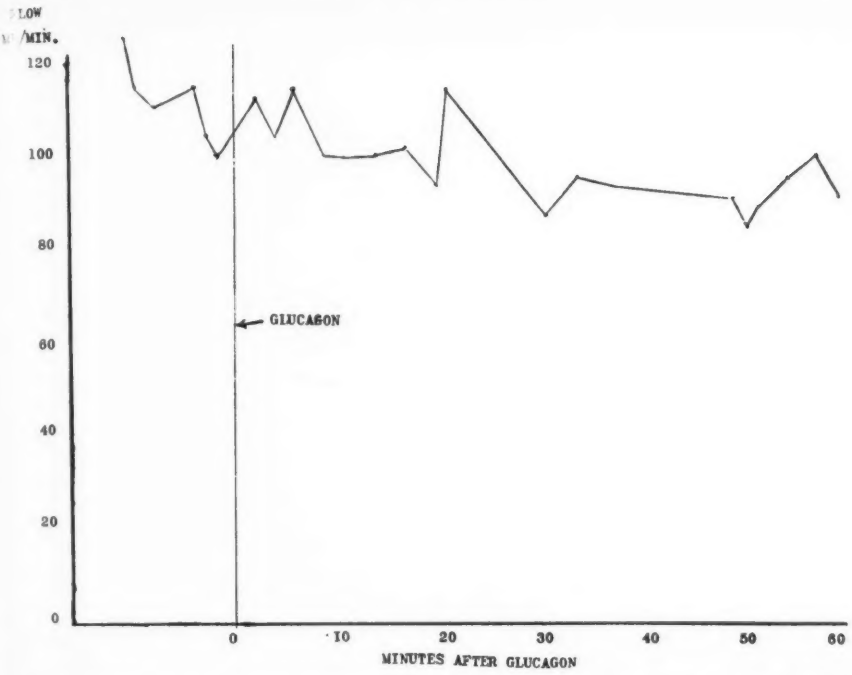

FIG. 2. The effect of glucagon on hindquarter blood flow in a dog which has been fasted for five days.

increased approximately 100% after glucagon, the hindquarter blood flow decreased to approximately 50% of baseline values. Although the hindquarter blood flow represents only about 10 to 15% of the cardiac output, it may also reflect the rate of blood flow (and the change in the rate of blood flow) occurring throughout the muscle mass of the body.

Although a direct action of glucagon on hindquarter blood flow cannot absolutely be ruled out at this time, this seems an unlikely explanation. That the hindquarter blood flow as well as the hepatic blood flow and hepatic glucose output response (8) were almost completely abolished when the animal was fasted for 5 days suggests the hindquarter blood flow response may be related to the hepatic response. We were unable to demonstrate a significant change in cardiac output after glucagon.

It is concluded that these flow alterations may represent a redistribution of the cardiac output incident to the hormone-induced hepatic glycogenolysis, rather than a direct vascular effect of the hormone, or the hyperglycemia produced by it.

The results of this study are in contrast to the results of Bondy, *et al.* (6), who reported no change in peripheral blood flow after glucagon administration in man. Although there is a species difference, it should be pointed out that Bondy's measurements were obtained with finger plethysmography; it need hardly be mentioned that the finger contains almost no muscle save for a slip of the lumbrical at the most proximal portion. Finger plethysmography (in addition to having inherent errors as a flow measurement) would not be a valid representation of the blood flow through the muscle mass of the

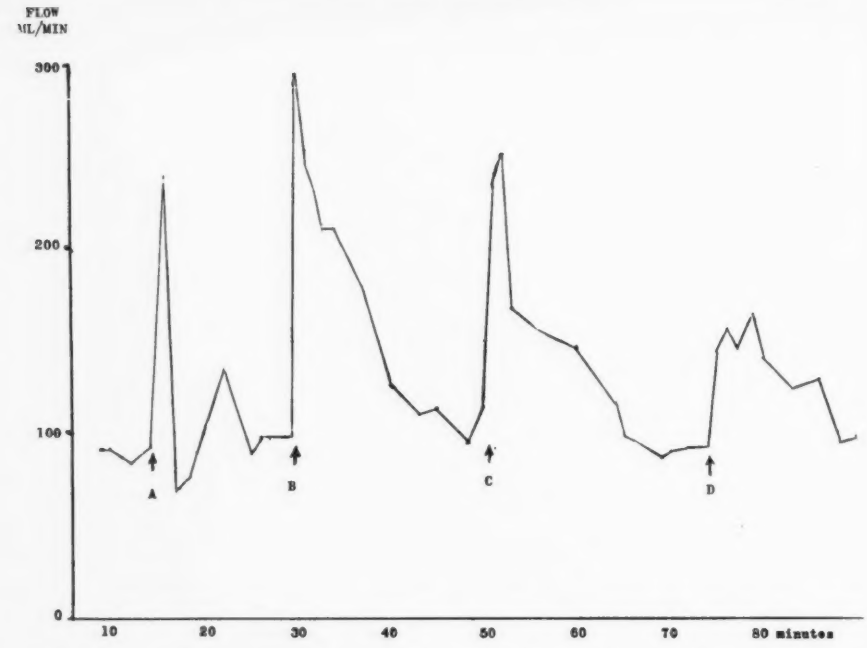

FIG. 3. The effect of saline or glucose solutions on hindquarter blood flow. The following solutions were added at the arrows:

A. 10 gm. glucose (20 cc. 50% solution) carotid artery catheter
B. 10 gm. glucose (200 cc. 5% solution) into jugular vein catheter
C. 200 cc. 0.9% saline into jugular vein catheter
D. 10 gm. glucose (20 cc. 50% solution) into femoral vein catheter

extremity. Extrapolation from finger plethysmography to the total peripheral blood flow in a larger vascular bed seems unwarranted.

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PHARMACOLOGICAL CHARACTERISTICS OF NEUROHYPOPHYSIAL HORMONES FROM A MARSUPIAL (*DIDELPHIS VIRGINIANA*) AND A MONOTREME (*TACHYGLOSSUS* (*ECHNIDNA*) *ACULEATUS*)[1]

ABSTRACT

The neurohypophysis of an opossum (*Didelphis virginiana*) and a whole pituitary from a spiny anteater (*Tachyglossus aculeatus*) were extracted and subjected to selected biological assays. The results suggest that these pituitaries contain arginine vasopressin and oxytocin. They resemble, therefore, the typical mammalian pituitary rather than that of reptiles or birds.

Arginine vasopressin appears to be the vasopressor-antidiuretic principle in the neurohypophyses of all placental mammals that have been studied with the exception of the pig, which has lysine vasopressin instead (1). The vasopressor and antidiuretic principle in neurohypophysial extracts from birds and reptiles differs from the vasopressins. It has pharmacological properties indistinguishable from those of arginine vasotocin (8-arginine oxytocin) (2, 3). This analogue of the mammalian neurohypophysial hormones was synthesized by Katsoyannis and du Vigneaud (4) and generously supplied to us for pharmacological study. We have tentatively identified arginine vasotocin in pituitary extracts from the chicken (2), a turtle (3) and a crocodilian, the caiman (unpublished). Its presence in species from three such divergent lines of reptilian evolution, as well as in amphibians (3), strongly suggests that this peptide was also present in the reptilian ancestors of the early mammals.

It is of interest, therefore, to investigate the neurohypophysial hormones of surviving non-placental mammals in an attempt to define more precisely the location in vertebrate phylogeny at which arginine vasopressin displaced arginine vasotocin as the vasopressor-antidiuretic principle of the neurohypophysis.

A single neurohypophysis from an adult male opossum (*Didelphis virginiana*) collected locally was dehydrated in acetone, desiccated, and extracted in 0.3% acetic acid by immersion for 5 minutes in boiling water. The extract was centrifuged and the supernatant was sealed in ampules and refrigerated. A similarly desiccated whole pituitary from an echidna (*Tachyglossus aculeatus*) was kindly prepared for us by Mrs. Margery Alltoft of the Department of Physiology, University of Melbourne, and mailed to us in a sealed ampule. The dry weight of this pituitary was 20.9 mg. It was ground and extracted in the same manner as the opossum neurohypophysis.

Both extracts were assayed by methods that are described elsewhere (1, 2, 5, 6). The results are shown in Table 1. The activities of synthetic arginine vasotocin, and of highly purified arginine vasopressin, lysine vasopressin, and oxytocin on these assays are included as bases for comparison.

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TABLE 1. ACTIVITIES OF OPOSSUM AND ECHIDNA EXTRACTS AND OF NEUROHYPOPHYSIAL HORMONES ON SELECTED ASSAYS

Preparation	Assays†			
	Rat vasopressor	Rat uterus (no magnesium)	Hen oviduct	Frog bladder
Opossum extract Units/gland	1.10 (0.92–1.35)	0.39 (0.32–0.48)	0.65 (0.47–0.88)	≪5
Echidna extract Units/gland	1.02 (0.86–1.19)	0.31 (0.25–0.38)	1.06 (0.96–1.17)	<2
Arginine vasotocin Units/mg.	64 (62–67)	37 (34–41)	640	10,000
Arginine vasopressin* Units/mg.	300	9	240	70
Lysine vasopressin* Units/mg.	200	5	15	<5
Oxytocin* Units/mg.	7	420	25	360

* Highly purified natural hormones kindly supplied by Dr. du Vigneaud and his co-workers. Higher absolute potencies have been observed in other preparations of these peptides.
† Figure in parentheses are 95% confidence limits.

Inspection of the relative activities of both extracts allows a tentative identification of the active principles. It is clear that neither extract could owe its vasopressor activity to arginine vasotocin since there is relatively low activity on the hen oviduct and frog bladder assays. On the other hand, the oxytocin that is believed to be present, as indicated by the rat uterus assay, is inadequate to explain the activity of either extract on the hen oviduct assay. If the hen oxytocic activity is due to a vasopressin it must be arginine vasopressin.

The marsupial and the monotreme pituitaries examined, therefore, appear to be typically mammalian in character, containing oxytocin and arginine vasopressin. This would indicate that arginine vasopressin made its appearance very early in mammalian evolution, either in the reptilian stock from which mammals descended or in a very early mammalian ancestor common to monotremes, marsupials and placental mammals.

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PERCY T. DIORETS, ENDOCRINOLOGIST—A FABLE[1]

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> "Just what are we doing here, spinning on a tilted planet swinging round a star?—We can look at the problem in two ways. What does a sun amount to without a living planet to nourish at its side, and what is a living being that we should pit its worth against a stellar furnace and the universe at large?"
>
> N. J. BERRILL[2]

MORE than two hundred years ago on a planet of a certain star an intelligent race of beings learned how to manufacture anti-matter in great quantity, but lacked effective means of keeping it apart from the natural kind. The complete release of nuclear energy that ensued exploded the entire solar system of the star and converted some of its substance into elementary particles.

In the year 1887, a neutron, spinning and twisting away from the cosmic explosion, passed through the left gonad of Orville Diorets, a clergyman in a small community of southern Iowa, as he bent over an ice cream freezer at a church social. The Reverend Diorets was unaware of the event which modified the chemistry of a gene in a single sperm cell. The mutation was not subsequently revealed in his son, who became a rural mail carrier, but his grandson, Percy T. Diorets, bore testimony to this rare event—a mutation which enhanced the ability of the brain to function creatively.

Percy T. Diorets, born in the 1930's, was, like the rest of us, highly improbable. He was to become a gifted scholar, but we cannot explain him solely in terms of his genetic history. At the fetal age of five months he was almost aborted. The fetus that was to be christened Percy struggled with a trace of purpose to remain sheltered. Either because of or in spite of his mother being treated with progesterone, an abortion did not occur, but this prenatal experience imprinted the mind of Percy T. Diorets with a fear of the outside world. And the outer world did indeed build upon this psychic trauma of prenatal life, first with the birth shock and again at the age of four months, when he developed an incestuous desire for a maiden aunt who made and sold hats. Such were the events which established hidden fears and wants in Percy, which in an Adlerian sense reinforced his drives to creativity.

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[1] Presidential address given at the 1960 meeting of the Endocrine Society.

[2] You and the Universe. Dodd, Mead & Co., New York, 1958.

The intellectual gifts of Percy T. Diorets were not unnoticed by his family and friends. As a youth he appeared briefly on a national television quiz show but was defeated by a taxi driver who had committed to memory the political platforms of both major parties for each presidential year. Scholarship aids came to Percy, and his undergraduate years mingled boredom with occasional inspiration by a great teacher. Happily, his interests were inner-directed, and he probed here and there into the great books of science and philosophy.

But at this time of youth Percy was not yet safely on the way to success. During his senior year of college he met and instantly loved Mrala Noitcaer. Mrala was a delight to the viscera. Her curves bespoke a perfect endocrine balance, her hair was like spun circus candy, her eyes were green and slightly wicked like a pubescent kitten's, her lips tasted like strawberry soda, and she had one brain cell. For a time Mrala responded instinctively to Percy as to any human male animal, but, alas, he could not meet the competition he faced from Alvin Sitset. Alvin was older than Percy. He ran a delicatessen for his aging father. Good fortune had avoided him until the day he discovered in the vegetable bin a rutabaga which closely resembled the great senator Wolbdnalley. Alvin appeared on a series of radio and television programs and was interviewed for his opinion on foreign policy. Fame appealed to Mrala, so she married Alvin.

On the rebound Percy became temporarily devoted to Olga Negordna, a muscular intellectual with hirsutism. Olga was secretary of a small, noisy group of student communists. Percy was not affected by this political philosophy, but he admired Olga because she was intelligent and professed to love humanity. He abandoned her upon discovering that she hated people.

As Percy ricocheted from this second encounter, another accident had an important effect upon his life. He was struck down in a corridor of the university hospital by a hot-food cart. A simple fracture of the tibia placed him in a hospital bed where he was comforted and held by a gentle, comely student nurse who had identified Percy as good husband material. She was a perfect mother image. They were married, and for the rest of his life Percy lived happily with her.

Since we are endocrinologists, let us digress for a moment and see what happened to Olga. Her endocrinopathy was diagnosed and successfully treated at the University of Iowa hospital, where physicians and some well known anatomists have done well at this sort of thing. Removal of gonads which did indeed contain interstitial cells, some plastic surgery and appropriate therapy with estrogens transformed Olga into a desirably shaped female who now abandoned her political interests. Seeking a more frivolous existence she transferred to the University of Wisconsin where, in the spring of her senior year, she became Queen of the May.

As did many of us, Percy T. Diorets searched his soul to make a choice

between a research career and that of a physician. Said his most revered teacher: "Man has no higher activities than these: the search for truth and the healing of the sick." But the basis for decision had been built into Percy's nature. When given a taste of research, his interest flared and burned brightly until it was smothered by his fellow scientists.

Percy applied for and was awarded a fellowship at another university to do graduate study under the great Professor Suopmop. The professor traveled much, he aided decisions at high levels, he made frequent pronouncements to the press, he could be found at or near the front of the academic procession at each commencement, but he was seldom in the laboratory. Only the oldest animal caretaker could remember when Professor Suopmop had done an experiment with his own hands.

Percy foundered for a time in the professor's lab, but perhaps it was good fortune that he was left alone. Certainly it was fortuitous when a medical student assigned to assist him happened to section the xyphoid process of a rat and inquire about the nature of some nests of non-cartilaginous cells he observed under the microscope. "These could be secretory cells," thought Percy. He removed the xyphoid process from each of a dozen rats and observed a fascinating result—the tail ceased to grow. A year of rewarding research followed. By the end of it Percy had an extract which, crude as it was, not only normalized tail growth in xyphoidectomized rats but stimulated tails to grow to abnormal lengths. He named the principle Xyloxin and reported the results for the first time to the great man. Professor Suopmop received the news with disbelief and then, since the evidence was a convincing tail, with great excitement. "I have been invited to address the Academy of Science Writers on current advances in endocrinology," he said. "May I tell them about this work?" Percy happily agreed and began to wait anxiously for news of the report. "Professor Suopmop Discovers New Hormone—Possible Key to Cause of Cancer," announced the headlines. Percy read the somewhat garbled reports. His name did not appear. The professor, when he returned, said, "I shall see to it that you are elected to Sigma Xi," and he took active charge of the project. When the results were published in a scientific journal, the name Diorets did appear with that of Suopmop, but somehow the latter continued to receive the credit. Percy was sometimes photographed with the professor. He was always identified as a student or a lab assistant. Under the direction of Professor Suopmop the work went badly for a time, but he brought into the team a competent biochemist and then organic chemists as the hormone was purified. Identification and synthesis followed. Honors came to the Professor in abundance. Percy gained only reflected glory. Somewhat bitter, he considered transferring to another institution, but it was Professor Suopmop himself who left, to become president of a great Foundation. "I am sorry to leave the laboratory," he said to Percy. "My pioneering days are over."

Percy, now possessing an advanced degree, remained in the laboratory as a research associate and then as an assistant professor. Again almost completely free to explore his own research interests, his gift for inquiry flourished, and in most seasons it flowered. These were the golden years during which he experienced the exquisite torture of the restless, inquiring mind.

But Percy T. Diorets was also human. His vague hunger for security, approbation, and power had been sharpened by closeness to the fame that had flirted with him. So he accepted the increased responsibilities that insidiously accrue to successful men as they grow older. He became an important scientist and eventually held a chair of biochemistry. Within his university he served on committees by the dozen. He became chairman of the committee on student parking. During an average year his various university committees considered 131 problems and solved 8 of them. The societies in which he held membership showed the same tendency to metastasize into committees. One society had 77 separate committees, including two on the organization and scheduling of committees.

Percy lived his life among others who were unlike our image of the ideal scientist. Some were publication mad; others, a smaller number, worked unceasingly to accumulate data which they never published. Some did not work at all, either because they had achieved security or because of vague or unconscious fears, they automatically rejected each research plan as untenable. There were many who suffered mental anguish and exhibited strong defensive behavior whenever a cherished belief was challenged. Percy shared with many of his fellows a drive for power over others, which led to intense and sometimes savage competition for material rewards and prestige. A lonely man, he longed for friendships that he did not know how to attract. He knew the following poem on friendship by Dinah Maria Muloch Craik, a poem which had been appreciated in earlier years by Fuller Albright, John Browne and Perry McCullagh.

> Oh, the comfort, the inexpressible comfort
> Of feeling safe with a person,
> Having neither to weigh thoughts
> Nor measure words,
> But pouring them all out
> Just as they are,
> Chaff and grain together;
> Certain that a faithful hand
> Will take them and sift them
> Keep what is worth keeping
> And with a breath of kindness,
> Blow the rest away.

Since he was a member of a medical school faculty, he watched year by year the always amazing metamorphosis of students into physicians and surgeons. The change was not complete in all. Some remained brash and

rude—never fully concealing their aggressions toward patients and colleagues. Others went on to specialize in diseases of the rich. But many became gentle, compassionate, and wise. Percy T. Diorets envied those who had learned to practice the art of science of healing.

Percy also became a consultant to various granting agencies. He learned the importance of linking each grant application to a disease and of proposing projects that promised to yield to investigation—and of never, never asking for funds to test a daring idea when there was obvious risk of failure. These had to be kept in the quiet of the scientist's own mind and studied behind the closed doors of the laboratory until the pilot experiment either failed or until the value of further study could be documented.

Percy understood that most of the great foundations and the granting agencies of government do appreciate the importance of freedom of inquiry but that collectively man has never learned how to deal efficiently with new ideas. He observed that some scientists, having mastered the art of persuasion, can, by claiming great discoveries and by promising much, obtain research funds for projects that could not be successfully defended by the average scientist. Many became big-time operators and strove mightily to become super-big-time operators. At this time in his life, Percy T. Diorets knew that the research operation, that aims for maximum size and frequent research reports, does not always achieve quality. He watched with misgivings the development of giant screening programs based on the philosophy: "Make a compound and then search for a disease that it will cure." He believed screening to have some worth, and it might by chance lead to cures of one or more important diseases. But sometimes he was distressed upon reflection that the cost of such programs, apart from the appropriations alone, included an inestimable loss as a consequence of the man-power diverted from basic research which might some day bring insight into the causes of the great diseases.

Percy did encourage a rational approach to the problems of disease in his own department. There was among his post-doctorate fellows a brilliant organic chemist who proposed the synthesis of a steroid having a side chain shaped like a crab. Why young Boris Cigol expected this compound to block the growth of cancer was not clear, but he had faith in the idea and he synthesized the compound—the year was 1984—and named the result Crabamine. Sure enough, it inhibited the growth of radiation-induced carcinomas in the sacred baboon which had replaced the mouse as the favorite experimental animal of cancer researchers. Professor Diorets was delighted when he learned of the results. "I have been invited to address the International Cancer Society Symposium in Rangoon," said he. "May I report your discovery to them?" Young Boris Cigol happily agreed. "Savant announces Cure for Cancer!" proclaimed the papers. Percy was at first deeply worried by the exaggerated news reports and also was concerned a bit by the fact that his young colleague was not receiving

credit for the work and was very hurt by the lack of recognition. Crabamine turned out indeed to have therapeutic value in treating cancer and was widely recognized as a great contribution, and credit continued to come to the older man. Percy did not intend to be unjust; his own early experience with Professor Suopmop still rankled. "But," thought he, "I gave this young man his chance in the laboratory. I stood by him when he was confused and clumsy. I started him on this general line of research, and I provided the place and funds for the research. I have given credit publicly to my colleague and have permitted him to publish as senior author. This young man is an ingrate."

Percy T. Diorets, already a well known scientist, was famous. Awards and honorary degrees came to him. He moved from his university post to become director of a great institute; he was now a super-big-time operator. Public relations and raising funds became his full-time job. He did work at research policy but relied heavily on committee planning. His institute did programmatic research. It was a productive program although no great discoveries came from it.

Among his most respected, and perhaps envied, fellow scientists was a great clinical and laboratory investigator who directed a small research institute. This man had but a half-dozen senior staff members, every one original and creative. Said the director to each member of his staff, "You have no duties here, only opportunities." The director did not direct; he believed in freedom of inquiry. With a business manager to free him from non-scientific activities, the director too spent his time in the laboratory.

Percy T. Diorets, a gifted scientist, was not a wise director of research, although he was moderately good. Power and recognition did have appeal for him, and he did not actively resist the forces that separated him from the laboratory in spite of knowing that inquiry itself is a higher form of human activity than is administration. Percy had heard this once as a young man.

As the years passed, Percy T. Diorets began to experience vague feelings of dissatisfaction with his life. He still received approbation from many sources, sometimes from men who feared his power or from others who sought his aid. There were enemies too—among them more than one former student. He was getting old, no denying that.

> "Ivy, ivy on the wall
> When your leaves begin to fall
> Do you find the will to cling
> Nourished by a dream of spring?"[3]

No, Percy did not really want to relive his life. At least he was not conscious of such a wish. But there was a feeling that he had not made the most of it and that opportunities for discovery would not come again.

[3] From unpublished verse by David J. Ingle.

Perhaps this was the male climacteric or even an "adrenopause"—he had heard the word frequently in his student days but almost never again.

* * *

We will not dwell on the aging and eventual demise of this great man who lived a sometimes happy, sometimes tortured life which seemed to have been influenced so frequently by chance. (Or is chance, as Pope has said, "Direction which thou canst not see"?) Let us instead consider the significance of his life.

If we reflect upon the size of the universe, the existence of human life or even of the small planet upon which we live seems of little moment. Our solar system is a part of a galaxy which is 100,000 light years in diameter. Our galaxy, the Milky Way, is one of a billion estimated to be within range of the light telescope. There is no compelling reason to assume that even the largest radio telescopes now planned will probe to the edges of the universe.

But if we examine the evolution of the universe, man does become important. There is mystery enough about the inorganic stuff of which the world is made and how it evolved into systems. But when systems became alive, the order of complexity became far greater than that of matter which does not live. There is so much to wonder about the nature of a single cell, how it grows and replenishes itself, how cells come to live together and to evolve specialization of functions. Of course some may prefer the cold beauty of the stars to that of a rose, but it is distance that lends enchantment to these great thermonuclear storms. The emergence of the nervous system, social organization, awareness, and finally of man himself, who can dream and plan and reflect on his nature and exhibit altruism, surely represents a higher and more important part of nature than the great masses of elementary particles and elements which make up so much of the universe. Man deserves respect for being alive—and some men greater respect for having become creative.

Percy T. Diorets was one of these. Society did not make the best possible use of his genius, but there was no better time before his for a gifted man to live. From the standpoint of evolutionary biology, we do not rant at the errors of nature or at the tooth-and-claw way that has not been completely lost from the human brain. Rather we wonder at the positive achievements of nature and its greatest gift of all—the capacity of man to control his own future even though error, chance, and risks are still a part of nature and the evolutionary process.

THE ENDOCRINE SOCIETY

CITATIONS FOR THE 1960 AWARDS

The Endocrine Medal

The Fred Conrad Koch Award is the honor of highest distinction conferred by the Endocrine Society. In presenting this medal to Professor Emil Witschi, the Society pays tribute to a man who is both a pioneer and active contributor to the field of sex embryology and endocrinology.

Born in an Alpine valley near Bern, Switzerland, Emil Witschi developed early a fascination for the variety of animals with whom he shared his pastoral surroundings. This interest in natural history was nurtured under the tutorial guidance of the classical embryologist, Richard Hertwig, with whom Professor Witschi prepared for his doctorate, awarded by the University of Munich in 1913. His indoctrination as a naturalist and his recognition of the importance of comparative biology in the understanding of embryologic and physiologic events have led him to range broadly in his investigations of gonadal and other endocrinologic problems. Contained in his abundant scientific bibliography are studies on more than 30 different animal forms, ranging from invertebrates to man and including such unusual creatures as the Western banded gecko, African bishop birds, the nine-banded Armadillo and the diamond-back terrapin. Few learned societies have the privilege of honoring a colleague with achievements so diversified as to include the identification of a sub-species of frog, description of ear development for underwater hearing, the analysis of nuptial plumage in African birds, and the study of sex behavior of herring gulls. The broad scope of his work is, itself, a tribute to his zealous scientific curiosity, pursued with endless vitality and with the highest degree of competence.

The name of Witschi is identified most intimately with the inductor theory of sex differentiation, formulated and proposed by him in 1914. In those early years his studies on the process of sex differentiation in Rana temporaria led him to the realization that all embryos and primordial germ cells are bipotential and that several factors, both genetic and nongenetic, decide the alternative of male or female differentiation. He demonstrated that the embryonic gonadal cortex is an inductor of female differentiation and the medulla an inductor of male differentiation. These concepts, formulated by Witschi before 1920 serve as a basis for the current re-evaluation of human intersexuality—a development in which he has played an active role.

In 1926 Professor Witschi took leave of his native Switzerland and his post as a lecturer at the University of Basel to visit the United States as a Rockefeller Foundation Fellow. He spent nearly two years in the Osborn

laboratory at Yale, with Carl Moore at Chicago and Herbert Evans at California. In each laboratory he found a stimulating environment and a great interest in the field of sex differentiation. With the emphasis on this area of research shifting from Europe to the United States, the decision to return to this country and assume American citizenship was a natural one for Professor Witschi and in this he was supported by his faithful wife Martha. That he has been forgiven his exodus from the Swiss scientific family is evidenced by his appointment, nearly twenty years ago, to the Swiss Academy of Sciences and by the honorary Doctor of Medicine degree conferred on him by the University of Basel on the occasion of the 500th anniversary of that historic Institution.

In America, Professor Witschi chose the beautiful setting of the State University of Iowa, located on the fertile rolling hills overlooking the Iowa river. His appointment in 1927 as Professor of Zoology was destined to add further luster to and to maintain the prominence in endocrine research of the department led by W. W. Swingle. Continuously, Professor Witschi and his growing school of Iowa students broadened the attack on the problem of sex differentiation to include the study of fishes, reptiles, birds and mammals. As a result, Witschi's inductor theory has found widespread acceptance as a fundamental principle in the development of all vertebrate gonads. Through the years, he has developed a truly remarkable laboratory for studies in comparative endocrinology. With these facilities it has been possible to study many hormonally controlled sexual characteristics that extend beyond those found in the usual species of laboratory animals. His studies on the comparative aspects of pituitary gonadotrophins provide important fundamentals for the current activity in this field. His analysis of the hormonal control of feather and bill coloration in birds reveals basic considerations bearing on the evolution of endocrine reactions. His work on temperature-effects and over-ripeness in amphibian eggs have been of fundamental importance in the fields of teratogenesis and fetal abnormalities. Among his numerous contributions to the study of genetics is included the discovery of the chromosomal mechanisms for sex inheritance in two different species of amphibia.

Throughout this lifetime of devotion to scientific research, Emil Witschi has fulfilled with great conscience his responsibilities as a member of the teaching faculty of a great State University. In 1955 he published his second book, an outstanding textbook, entitled, *The Development of Vertebrates*. Replete with original concepts and data, it represents an important contribution to the teaching of embryology. The first book under Witschi's authorship, also prepared for students, appeared nearly 40 years earlier when, as a teacher in the Science *Gymnasium* of Basel, he wrote and illustrated a delightful volume entitled, *The Land Animals*. Intended to inspire in his young students an interest in biology, this book served also as an outlet for his artistic inclinations. With this volume, Witschi purged him-

self of a lingering idea to foresake science in favor of a career in the graphic arts. Those who have observed his classroom lectures, which might well serve as sketching lessons in a school of fine arts, will recognize that he has succeeded in pursuing both courses.

By virtue of the achievements that have been cited and numerous others that are omitted here for the sake of brevity, Emil Witschi is amply qualified to receive this coveted award, Not to be omitted, however, is recognition of his invaluable service to the field of endocrinology through the inspiration he has provided to young scientists. He has advised and guided over forty men and women to the Ph.D. degree and many other young investigators whose researches border on his interests have profited from his consultation, encouragement and suggestions. This boundless fervor to broaden the horizon of scientific understanding characterizes Emil Witschi. His continuing activity in research and teaching will contribute toward a further expansion of this horizon.

Ciba Award

Dr. Don Harry Nelson:

The Endocrine Society is pleased to present to you the 1960 Ciba Award.

This is given in acknowledgment of your distinguished contributions to man's knowledge of the functions of the adrenal cortex. The precision and incisiveness of these highly original studies have established you as the one of most gifted young investigators in endocrinology.

You have provided a chemical technique for the direct study of the circulating adrenal hormones. You have devised a method for obtaining hormone-rich blood directly from the adrenal gland and have shown that the hormone-content of such blood collected close to the gland greatly exceeds that found in the peripheral blood. This finding alters our concept of the "milieu interieur" as a homogenous invironment and shows that this internal environment has within it many internal pools and eddies.

You have developed a method which permits the assessment of the amount of ACTH in peripheral blood, thus adding much to our ability to analyze the basic mechanisms involved in disease states affecting the pituitary-adrenal axis.

You have participated in vital phases of the earliest studies on the non-hormonal agents capable of suppressing adrenocortical function in animals and man.

You have described the development of pituitary tumors in previously adrenalectomized patients and have characterized their endocrine and metabolic effects upon the host.

Careful observer, able investigator, competent physician and amiable colleague—we hail you and commend you and in affection and esteem we herewith present to you the Endocrine Society's Ciba Award for 1960.

Upjohn Scholar

The Upjohn Scholar for 1960 is Charles R. Kleeman. Dr. Kleeman was born in Los Angeles, California on August 19, 1923. He obtained his collegiate training at the University of California, and he was granted the B.S. degree from that institution in 1944, and the M.D. from the University of California School of Medicine in 1947. Dr. Kleeman has held the following positions: Intern, University of California Service, San Francisco Hospital 1947–48; Assistant Resident in Pathology, Mallory Institute, Boston City Hospital, 1948–49; Intermediate Resident in Medicine, Newington Veterans Administration Hospital, and Fellow in Metabolism, Yale University 1950–51.

Associate Chief, Biochemistry Unit. Q.M. Climatic Research Laboratory, Lawrence, Massachusetts, 1851–53; Lieutenant, Medical Corps, U.S. Army, 1951–53; Instructor in Medicine, 1953–55, Assistant Professor 1955–56, Metabolic Section, Department of Medicine, Yale University School of Medicine; Associate Clinical Professor of Medicine, University of California Medical School, Los Angeles, 1956–58; Chief, Metabolic Section, Veterans Administration Hospital, Los Angeles, California 1956 to present; Associate Professor of Medicine, University of California Medical Center, Los Angeles, 1959 to present.

During the last ten years, Dr. Kleeman has published more than fifty reports concerned with the role of the thyroid, parathyroids, adrenal cortex and neurohypophysis in controlling water and electrolyte metabolism in human beings and experimental animals.

During the year beginning July 1960, Dr. Kleeman will be on sabbatical leave. He will continue his investigations on water metabolism in the laboratory of Dr. Hugh Dawson, Department of Physiology, University College, London, England. The Upjohn Scholarship will be used by Dr. Kleeman for travel, and for support of his research program in England.

A Citation Presented at The Endocrine Society Testimonial Dinner in Honor of Roy Graham Hoskins

Presented to Roy Graham Hoskins, Ph.D., M.D. in recognition of his outstanding contributions to the advancement and dissemination of knowledge of the glands of internal secretion and in appreciation of his great labors on behalf of The Endocrine Society.

A pioneer in the unfolding role of the endocrines in man's physical and mental well-being, he helped found a science and give substance to a bright new epic in medicine. The sound guidance he gave to this field, especially during its formative period, was of crucial importance. To these endeavors he brought a keen and critical mind, an indomitable courage, boundless energy, a cheerful spirit and farsighted wisdom.

Roy Hoskins rallied interest to form in 1917 the Association for the Study of Internal Secretions, later to become The Endocrine Society. In the course of long and invaluable service to this Society he gave helmsmanship to its destinies as president, councilor and committeeman. He ventured the inauguration of a new journal, ENDOCRINOLOGY, serving as its editor for twenty-five years, and, in addition, significantly influenced the founding of *The Journal of Clinical Endocrinology*. Striving skillfully and often singlehandedly against lingering perversities, he handed on to future generations of endocrinologists two journals of distinction in the world of science and medicine.

To Roy G. Hoskins, who has served his field faithfully and well as scientist, author, editor, historian, teacher, friend and wise counselor, The Endocrine Society hereby does honor, proudly and with enduring gratitude.

1961 ANNUAL MEETING

The next annual meeting of The Endocrine Society will be held in the Hotel Biltmore, New York, N.Y. June 22–24, 1961.

ANNOUNCEMENTS

NOTICE

The Endocrinology Study Section, National Institutes of Health, has the following highly purified radioactive steroids available for distribution free to qualified investigators:

Material	*Specific Activity*
7-^{3}H Aldosterone	20 μc./μg.
7-^{3}H Cortisol	28 μc./μg.

Workers may obtain supplies of these steroids by writing to:

Dr. R. T. Hill, Executive Secretary
Endocrinology Study Section
Division of Research Grants
National Institutes of Health
Bethesda 14, Maryland

describing briefly the proposed work and giving an estimate of the amount of steroids required. Requests are filled as promptly as possible after review by a subcommittee. These steroids are prepared and distributed under a program generously supported by the National Institutes of Health.

These materials are stored at Tracerlab, Inc., Waltham, Massachusetts. They are dispensed in sealed glass ampules containing 30 μc. each, and in a solution of benzene and methanol.

Shipments will be made from Tracerlab, Inc., and the recipient will be billed by them, at the rate of $5.00 per ampule for handling and mailing.

WORCESTER FOUNDATION FOR EXPERIMENTAL BIOLOGY

In recognition of the world-wide need for scientific training in reproductive physiology, the Worcester Foundation for Experimental Biology

plans to initiate on January 1, 1961, a postdoctoral program in this field under a grant from the Population Council.

Fellowships for the program will be awarded to candidates possessing the Ph.D., M.D., or their equivalents. These Fellowships will carry a stipend of $5,500 per annum and will be for a twelve month period. An allotment will also be made for round-trip travel to Shrewsbury, Massachusetts. Application blanks may be secured from the Research Director of the Worcester Foundation for Experimental Biology, and should be returned at an early date. They will be processed by a selection committee of experts, and the Fellowship awards for the first year will be announced late in 1960. Applicants for participation in the program in 1962 and later years may communicate with the Director at any time, but their applications will not be acted upon in 1960.

The program will consist of a course of lectures and laboratory work. During the latter part of the year each Fellow will have the opportunity to participate in a research project pertinent to his interests and within the capacity of the program.

The lecture series will be given by the Program Director, various members of the staff of the Worcester Foundation, and guest experts. The lectures and accompanying laboratory work will cover various phases of reproductive processes, particularly in mammals. Subjects to be treated will involve a review of reproductive processes in the male, including consideration of spermatogenesis, hormonogenesis, the control of testis function, and the functional endocrinology of testis hormones. Extensive attention will be given to oogenesis, ovulation, and fertilization in the female, factors affecting the entire course of pregnancy and embryonic development, as well as the postnatal physiology of the female. Additional topics will include the role of extra-reproductive tract organs in reproductive processes, genetic and nutritive factors in reproduction, and the control of fertility and sterility.

Laboratory exercises will include the study of characteristic reproductive phenomena in various species, analytic procedures employed in determining the chemistry of reproductive organs, the biochemistry of spermatozoa and ova, and hormone analyses pertinent to gonad function, to sex cycles, to gestation and lactation.

TERATOLOGY SOCIETY

For several years scientists interested in basic problems of congenital malformations have held informal conferences in which questions of common interest were discussed. Anatomists, biochemists, embryologists, geneticists, obstetricians, pathologists, pediatricians, plastic surgeons and others attended these conferences which were in part supported by the Association for the Aid of Crippled Children, New York, N. Y., and the Human Embryology and Development Study Section of the National Institutes of Health. With the increased interest in recent years in this

area, it was felt that there was a need for a Society to hold regular meetings in which investigations concerned with etiology and morphogenesis of congenital malformations could be presented and discussed. Following the fourth teratology conference, which was held at the Memorial Sloan-Kettering Cancer Center in New York City and attended by 76 scientists from Canada, England, France, Germany and the U.S.A., "The Teratology Society" was formed for the purposes outlined above. The following officers were elected: President: Josef Warkany, M.D., Cincinnati, O.; President-Elect: James G. Wilson, Ph.D., Gainesville, Fla.; Secretary-Treasurer: Marjorie M. Nelson, Ph.D., San Francisco, Calif.; Recorder: Sidney Q. Cohlan, M.D., New York, N. Y.; Council: F. Clarke Fraser, Ph.D., M.D., Montreal, Canada, David L. Gunberg, Ph.D., Portland, Ore., and M. Lois Murphy, M.D., New York, N. Y. The National Foundation assisted in the formation of the Society with advice and financial aid. Inquiries about The Tetratology Society should be directed to Dr. Marjorie M. Nelson, Department of Anatomy, School of Medicine, University of California, San Francisco 22, Calif.

AMERICAN DAIRY SCIENCE ASSOCIATION

The Effect of Germ Cell Damage on Animal Reproduction

Papers of
The Fourth Biennial Symposium on Animal Reproduction
will be published as a
Supplement to *Journal of Dairy Science*
Volume 43, April, 1960

This 168-page issue consisting of eight papers by internationally recognized authorities will be available in May, 1960, at a postpaid price of $2.50 per copy. Payment to be sent with order to

H. F. Judkins
32 Ridgeway Circle
White Plains, N. Y.

6TH INTERNATIONAL CONGRESS OF INTERNAL MEDICINE

The 6th International Congress of Internal Medicine will be held in Basel, Switzerland, August 24–27, 1960.

All correspondence should be addressed to:

Sekretariat
6. Internationaler Kongress für Innere Medizin
Steinentorstrasse 13
Basel (Schweiz)